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REMARKS

Pending Claims

Claims 1-18, 20, 23, and 29 are currently pending. The Examiner has noted that claims 3-16, 20, 23, 28, and 29 have been withdrawn from consideration. However, Applicants respectfully remind the Examiner that claims 4, 5, 19, 21, 22, 24-27, and 30-45 were cancelled at the time that the application was filed. Accordingly, the claims withdrawn from consideration are 3, 6-16, 20, 23, 28 and 29. Claims 1-2 and 17-18 are currently under examination. Applicants submit that the cancelled claims were included in the application as filed in the interest of providing notice to the public of certain specific subject matter intended to be claimed, and were intended to be cancelled at the time of filing this application in the interest of reducing filing costs. Applicants expressly state that these claims were not cancelled for reasons related to patentability, and are in fact fully supported by the specification as filed. Applicants expressly reserve the right to reinstate these claims, or to add other claims during prosecution of this application, or a continuation or divisional application. Applicants expressly do not disclaim the subject matter of any invention disclosed herein which is not set forth in the instantly filed claims.

Elections and Restrictions

In Item 1 of the outstanding Office Action, the Examiner acknowledges Applicants' election with traverse of Group I in Applicants' Response to Restriction Requirement filed October 21, 2002, but maintains the requirement and makes it final, asserting that the examination of additional groups would prove to be a burdensome search. Applicants again respectfully remind the Examiner that the method claims of Groups III, VII, and X of the Restriction Requirement (i.e., claims 9-10, 20, and 23) are entitled to rejoinder upon the allowance of a product claim per the Commissioner's Notice in the Official Gazette of March 26, 1995, entitled "Guidance on Treatment of Product and Process Claims in light of *In re* Ochiai, *In re* Brouwer and 35 U.S.C. § 103(b)" which sets forth the rules, upon allowance of product claims, for rejoinder of process claims covering the same scope of products. See also M.P.E.P. 821.04.

Support for the Amendments

This amendment incorporates explicitly disclosure implicitly present, based on, e.g. disclosure in the specification. Claim 1 has been amended so that part (d) now reads, "an immunogenic fragment of a polypeptide having an amino acid sequence of SEQ ID NO:1, said fragment comprising at least 15 contiguous amino acid residues." No new matter has been added by this amendments. Support for this amendment may be found in the Specification at p. 51, line 1. Entry of the amendment is respectfully requested.

The Rejection

Claims 1, 2, 17 and 18 stand rejected under 35 U.S.C. § 101 based on the allegation that the claimed invention lacks patentable utility. The rejection alleges in particular that:

- there is no well-established, specific and substantial utility for the claimed polypeptide as different stomatin-like proteins would have different functions and the skilled artisan would have to determine the function of this particular polypeptide in order to determine how to use it.
- the claimed invention is not supported by either a substantial and specific asserted utility or a well established utility. The specification discloses no uses for the broadly claimed polypeptides. A specific utility is one that is particular to the subject matter claimed, while a substantial utility is one that defines a "real world" use. Utilities that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use are not substantial utilities.

Utility Rejection – 35 USC § 101

The rejection of claims 1, 2, 17 and 18 is improper, as the inventions of those claims have a patentable utility as set forth in the instant specification, and/or a utility well-known to one of ordinary skill in the art.

The invention at issue, identified in the patent application as novel integral membrane protein, abbreviated as IMP, is a polypeptide sequence encoded by a gene that is expressed in prostate tumor, breast tumor, and pancreatic tumor tissues of humans. The novel polypeptide is demonstrated in the

specification to be a member of the class of integral membrane proteins, whose biological functions include the regulation of ion channel activity (Specification at p. 3, lines 12-21). As such, the claimed invention has numerous practical, beneficial uses in toxicology testing, drug development, and the diagnosis of disease, none of which require knowledge of how the polypeptide actually functions. The claimed invention also can be used as tissue or tumor marker (Specification at p. 15, lines 12-16).

The fact that the claimed polypeptide is a member of the integral membrane protein family alone demonstrates utility. Each of the members of this class, regardless of their particular functions, are useful. There is no evidence that any member of this class of polypeptides, let alone a substantial number of them, would not have some patentable utility. It follows that there is a more than substantial likelihood that the claimed polypeptide also has patentable utility, regardless of its actual function. The law has never required a patentee to prove more.

There is, in addition, direct proof of the utility of the claimed invention. Applicants submit with this response the unexecuted Declaration of Furness (executed version will be forwarded to the Examiner as soon as possible) describing some of the practical uses of the claimed invention in gene and protein expression monitoring applications as they would have been understood at the time of the patent application. The Furness Declaration describes, in particular, how the claimed polypeptide can be used in protein expression analysis techniques such as 2-D PAGE gels and western blots. Using the claimed invention with these techniques, persons of ordinary skill in the art can better assess, for example, the potential toxic effect of a drug candidate. (Furness Declaration ¶12 (b)).

The Examiner contends that the claimed polypeptide cannot be useful without precise knowledge of its function. But the law never has required knowledge of biological function to prove utility. It is the claimed invention's uses, not its functions, that are the subject of a proper analysis under the utility requirement.

In any event, as demonstrated by the Furness Declaration, the person of ordinary skill in the art can achieve beneficial results from the claimed polypeptide in the absence of any knowledge as to the precise function of the protein. The uses of the claimed polypeptide for gene expression monitoring applications including toxicology testing are in fact independent of its precise function.

The Office Action is replete with arguments made and positions taken in a misplaced attempt to justify the rejections of the claims under 35 U.S.C. §§ 101 and 112. The Examiner's positions and arguments include that the invention (i.e., the SEQ ID NO:1 polypeptide) is not supported by either a "specific asserted utility" or a "well established utility" (p. 3 Item 6), "...does not teach a relationship to any specific disease or establish any involvement in the etiology of any specific disease" (p. 3 Item 6, also p. 6, first paragraph) or is otherwise insufficient to constitute substantial, specific and credible utilities for the SEQ ID NO:1 polypeptide (Office Action, e.g., p. 6).

Under the circumstances, Applicants are submitting with this Response a Declaration of Furness under 37 C.F.R. § 1.132 (the Furness Declaration). As we will show, the Furness Declaration shows the many substantial reasons why the Examiner's positions and arguments with respect to the use of the SEQ ID NO:1 polypeptide are without merit.

I. The Applicable Legal Standard

To meet the utility requirement of sections 101 and 112 of the Patent Act, the patent applicant need only show that the claimed invention is "practically useful," *Anderson v. Natta*, 480 F.2d 1392, 1397, 178 USPQ 458 (CCPA 1973) and confers a "specific benefit" on the public. *Brenner v. Manson*, 383 U.S. 519, 534-35, 148 USPQ 689 (1966). As discussed in a recent Court of Appeals for the Federal Circuit case, this threshold is not high:

> An invention is "useful" under section 101 if it is capable of providing some identifiable benefit. See *Brenner v. Manson*, 383 U.S. 519, 534 [148 USPQ 689] (1966); *Brooktree Corp. v. Advanced Micro Devices, Inc.*, 977 F.2d 1555, 1571 [24 USPQ2d 1401] (Fed. Cir. 1992) ("to violate Section 101 the claimed device must be totally incapable of achieving a useful result"); *Fuller v. Berger*, 120 F. 274, 275 (7th Cir. 1903) (test for utility is whether invention "is incapable of serving any beneficial end").

Juicy Whip Inc. v. Orange Bang Inc., 51 USPQ2d 1700 (Fed. Cir. 1999).

While an asserted utility must be described with specificity, the patent applicant need not demonstrate utility to a certainty. In *Stiftung v. Renishaw PLC*, 945 F.2d 1173, 1180, 20 USPQ2d 1094 (Fed. Cir. 1991), the United States Court of Appeals for the Federal Circuit explained:

> An invention need not be the best or only way to accomplish a certain result, and it need only be useful to some extent and in certain applications: "[T]he fact that an invention has only limited utility and is only operable in certain applications is not grounds for finding lack of utility." *Envirotech Corp. v. Al George, Inc.*, 730 F.2d 753, 762, 221 USPQ 473, 480 (Fed. Cir. 1984).

The specificity requirement is not, therefore, an onerous one. If the asserted utility is described so that a person of ordinary skill in the art would understand how to use the claimed invention, it is sufficiently specific. *See Standard Oil Co. v. Montedison, S.p.a.*, 212 U.S.P.Q. 327, 343 (3d Cir. 1981). The specificity requirement is met unless the asserted utility amounts to a "nebulous expression" such as "biological activity" or "biological properties" that does not convey meaningful information about the utility of what is being claimed. *Cross* v. *Iizuka*, 753 F.2d 1040, 1048 (Fed. Cir. 1985).

In addition to conferring a specific benefit on the public, the benefit must also be "substantial." *Brenner*, 383 U.S. at 534. A "substantial" utility is a practical, "real-world" utility. *Nelson v. Bowler*, 626 F.2d 853, 856, 206 USPQ 881 (CCPA 1980).

If persons of ordinary skill in the art would understand that there is a "well-established" utility for the claimed invention, the threshold is met automatically and the applicant need not make any showing to demonstrate utility. Manual of Patent Examination Procedure at § 706.03(a). Only if there is no "well-established" utility for the claimed invention must the applicant demonstrate the practical benefits of the invention. *Id.*

Once the patent applicant identifies a specific utility, the claimed invention is presumed to possess it. *In re Cortright*, 165 F.3d 1353, 1357, 49 USPQ2d 1464 (Fed. Cir. 1999); *In re Brana*, 51 F.3d 1560, 1566; 34 USPQ2d 1436 (Fed. Cir. 1995). In that case, the Patent Office bears the burden of demonstrating that a person of ordinary skill in the art would reasonably doubt that the asserted utility could be achieved by the claimed invention. *Id.* To do so, the Patent Office must provide evidence or sound scientific reasoning. *See In re Langer*, 503 F.2d 1380, 1391-92, 183 USPQ 288 (CCPA 1974). If and only if the Patent Office makes such a showing, the burden shifts to the applicant to provide rebuttal evidence that would convince the person of ordinary skill that there is sufficient proof of utility. *Brana*, 51 F.3d at 1566. The applicant need only prove a "substantial likelihood" of utility; certainty is not required. *Brenner*, 383 U.S. at 532.

II. Toxicology testing, use as a tissue or tumor marker, regulation of membrane conductance, and regulation of ion channel activity are sufficient utilities under 35 U.S.C. §§ 101 and 112, first paragraph

The claimed invention meets all of the necessary requirements for establishing a credible utility under the Patent Law: There are "well-established" uses for the claimed invention known to persons of ordinary skill in the art, and there are specific practical and beneficial uses for the invention disclosed in the patent application's specification. These uses are explained, in detail, in the Furness Declaration accompanying this response. Objective evidence, not considered by the Patent Office, further corroborates the credibility of the asserted utilities.

A. The claimed polypeptide's membership in the integral membrane protein family demonstrates utility

Because there is a substantial likelihood that the claimed IMP is a member of the family of polypeptides known as integral membrane proteins, the members of which are indisputably useful, there is by implication a substantial likelihood that the claimed polypeptide is similarly useful. Applicants need not show any more to demonstrate utility. *In re Brana*, 51 F.3d at 1567.

It is undisputed that the claimed polypeptide is a protein having the sequence shown as SEQ ID NO:1 in the patent application and referred to as IMP in that application. Applicants have demonstrated by more than reasonable probability that IMP is a member of the integral membrane protein family, and that the integral membrane protein of proteins includes stomatin and stomatin-like proteins, each of which regulate membrane conductance. IMP has structural homology with stomatin.

The Examiner must accept Applicants' assertion that the claimed polypeptide is a member of integral membrane protein family and that utility is credible to a reasonable probability unless the Examiner can demonstrate through evidence or sound scientific reasoning that a person of ordinary skill in the art would doubt utility. *See In re Langer*, 503 F.2d 1380, 1391-92, 183 USPQ 288 (CCPA 1974). The Examiner has not provided sufficient evidence or sound scientific reasoning to the contrary.

Nor has the Examiner provided any evidence that any member of the integral membrane protein family, let alone a substantial number of those members, is not useful. In such circumstances the only

reasonable inference is that the claimed polypeptide must be, like the other members of the integral membrane protein family, useful.

B. The Office Action failed to demonstrate that a person of ordinary skill in the art would reasonably doubt the utility of the claimed invention

Based principally on citations to scientific literature identifying some of the difficulties involved in predicting protein function, the Office Action rejected the pending claims on the ground that the Applicants cannot impute utility to the claimed invention based on the homology between the encoded polypeptide, IMP, and another polypeptide. The Office Action's rejection is both incorrect as a matter of fact and as a matter of procedural law.

While the Office Action has cited literature identifying some of the difficulties that may be involved in predicting protein function, none suggests that functional homology cannot be inferred by a reasonable probability in this case. Importantly, none contradicts Bork's later findings that there is a 70% accuracy rate for bioinformatics-based predictions in general, and a 90% accuracy rate for the prediction of functional features by homology. Bork, Genome Research 10:398-400 (2000). At most, these articles individually and together stand for the proposition that it is difficult to make predictions about function with certainty. The standard applicable in this case is not, however, proof to certainty, but rather proof to reasonable probability.

The literature cited in the Office Action may show that Applicants cannot prove function by homology with **certainty**, but Applicants need not meet such a rigorous standard of proof. Under the applicable law, once the Applicants demonstrate a *prima facie* case of homology, the Office must accept the assertion of utility to be true unless the Office comes forward with evidence showing a person of ordinary skill would doubt the asserted utility could be achieved by a reasonable probability. See *In re Brana*, 51 F.3d at 1566; *In re Langer*, 503 F.2d 1380, 1391-92, 183 USPQ 288 (CCPA 1974). The Office has not made such a showing and, as such, the Office Action's rejection should be withdrawn.

C. The uses of IMP for toxicology testing, drug discovery, and disease diagnosis are practical uses that confer "specific benefits" to the public

The claimed invention has specific, substantial, real-world utility by virtue of its use in toxicology testing, drug development and disease diagnosis through gene expression profiling. These uses are explained in detail in the accompanying Furness Declaration, the substance of which is not rebutted by the Patent Examiner. There is no dispute that the claimed invention is in fact a useful tool in two-dimensional polyacrylamide gel electrophoresis ("2-D PAGE") analysis and western blots used to monitor protein expression and assess drug toxicity.

The instant application is a continuation application of and claimed priority to United States patent application Serial No. 09/095,351 filed on June 9, 1998 (hereinafter "the Hillman '351 application"), which in turn was a divisional application of and claimed priority to United States patent application Serial No. 08/781,562 filed on January 9, 1997 (hereinafter "the Hillman '562 application"), all having the identical specification.

In his Declaration, Mr. Furness explains the many reasons why a person skilled in the art who read the Hillman '562 application on January 9, 1997 would have understood that application to disclose the claimed polypeptide to be useful for a number of gene and protein expression monitoring applications, *e.g.*, in 2-D PAGE technologies, in connection with the development of drugs and the monitoring of the activity of such drugs. (Furness Declaration at, e.g., ¶¶11-13). Much, but not all, of Mr. Furness' explanation concerns the use of the claimed polypeptide in the creation of protein expression maps using 2-D PAGE.

2-D PAGE technologies were developed during the 1980's. Since the early 1990's, 2-D PAGE has been used to create maps showing the differential expression of proteins in different cell types or in similar cell types in response to drugs and potential toxic agents. Each expression pattern reveals the state of a tissue or cell type in its given environment, *e.g.*, in the presence or absence of a drug. By comparing a map of cells treated with a potential drug candidate to a map of cells not treated with the candidate, for example, the potential toxicity of a drug can be assessed. (Furness Declaration at ¶11.)

The claimed invention makes 2-D PAGE analysis a more powerful tool for toxicology and drug efficacy testing. A person of ordinary skill in the art can derive more information about the state or states or tissue or cell samples from 2-D PAGE analysis with the claimed invention than without it. As Mr. Furness explains:

> In view of the Hillman '562 application, the Wilkins article, and other related pre-January 1997 publications, persons skilled in the art on January 9, 1997 clearly would have understood the Hillman '562 application to disclose the SEQ ID NO:1 polypeptide or the antibody to SEQ ID NO:1 polypeptide to be useful in 2-D PAGE analyses for the development of new drugs and monitoring the activities of drugs for such purposes as evaluating their efficacy and toxicity . . . . (Furness Declaration, ¶10)
>
> * * *
>
> Persons skilled in the art would appreciate that a 2-D PAGE map that utilized the SEQ ID NO:1 polypeptide sequence would be a more useful tool than a 2-D PAGE map that did not utilize this protein sequence in connection with conducting protein expression monitoring studies on proposed (or actual) drugs for treating cancers for such purposes as evaluating their efficacy and toxicity. (Furness Declaration, ¶12)

Mr. Furness' observations are confirmed in the literature published before the filing of the patent application. Wilkins, for example, describes how 2-D gels are used to define proteins present in various tissues and measure their levels of expression, the data from which is in turn used in databases:

> For proteome projects, the aim of [computer-aided 2-D PAGE] analysis . . . is to catalogue all spots from the 2-D gel in a qualitative and if possible quantitative manner, so as to define the number of proteins present and their levels of expression. Reference gel images, constructed from one or more gels, for the basis of two-dimensional gel databases. (Wilkins, Tab 1, p. 26).

D. The use of proteins expressed by humans as tools for toxicology testing, drug discovery, and the diagnosis of disease is now "well-established"

The technologies made possible by expression profiling using polypeptides are now well-established. The technical literature recognizes not only the prevalence of these technologies, but also their unprecedented advantages in drug development, testing and safety assessment. These technologies include toxicology testing, as described by Furness in his Declaration.

Toxicology testing is now standard practice in the pharmaceutical industry. See, *e.g.*, John C. Rockett, et. al., Differential gene expression in drug metabolism and toxicology: practicalities, problems, and potential, Xenobiotica 29:655-691 (July 1999) (Reference No. 2):

> Knowledge of toxin-dependent regulation in target tissues is not solely an academic pursuit as much interest has been generated in the pharmaceutical industry to harness this technology in the early identification of toxic drug candidates, thereby shortening the developmental process and contributing substantially to the safety assessment of new drugs. ((Reference No. 2), page 656)

To the same effect are several other scientific publications, including Emile F. Nuwaysir, et al., Microarrays and Toxicology: The Advent of Toxicogenomics, Molecular Carcinogenesis 24:153-159 (1999) (Reference No. 3); Sandra Steiner and N. Leigh Anderson, Expression profiling in toxicology -- potentials and limitations, Toxicology Letters 112-13:467-471 (2000) (Reference No. 4).

The more genes – and, accordingly, the polypeptides they encode -- that are available for use in toxicology testing, the more powerful the technique. Control genes are carefully selected for their stability across a large set of array experiments in order to best study the effect of toxicological compounds. See attached email from the primary investigator of the Nuwaysir paper, Dr. Cynthia Afshari, to an Incyte employee, dated July 3, 2000, as well as the original message to which she was responding (Reference No. 5) Thus, there is no expressed gene which is irrelevant to screening for toxicological effects, and all expressed genes have a utility for toxicological screening.

In fact, the potential benefit to the public, in terms of lives saved and reduced health care costs, are enormous. Recent developments provide evidence that the benefits of this information are already beginning to manifest themselves. Examples include the following:

- In 1999, CV Therapeutics, an Incyte collaborator, was able to use Incyte gene expression technology, information about the structure of a known transporter gene, and chromosomal mapping location, to identify the key gene associated with Tangier disease. This discovery took place over a matter of only a few weeks, due to the power of these new genomics technologies. The discovery received an award from the American Heart Association as one of the top 10 discoveries associated with heart disease research in 1999.

- In an April 9, 2000, article published by the Bloomberg news service, an Incyte customer stated that it had reduced the time associated with target discovery and validation from 36 months to 18 months, through use of Incyte's genomic information

database. Other Incyte customers have privately reported similar experiences. The implications of this significant saving of time and expense for the number of drugs that may be developed and their cost are obvious.

- In a February 10, 2000, article in the *Wall Street Journal*, one Incyte customer stated that over 50 percent of the drug targets in its current pipeline were derived from the Incyte database. Other Incyte customers have privately reported similar experiences. By doubling the number of targets available to pharmaceutical researchers, Incyte genomic information has demonstrably accelerated the development of new drugs.

Because the Patent Examiner failed to address or consider the "well-established" utilities for the claimed invention in toxicology testing, drug development, and the diagnosis of disease, the Examiner's rejections should be overturned regardless of their merit.

E. Objective evidence corroborates the utilities of the claimed invention

There is in fact no restriction on the kinds of evidence a Patent Examiner may consider in determining whether a "real-world" utility exists. "Real-world" evidence, such as evidence showing actual use or commercial success of the invention, can demonstrate conclusive proof of utility. *Raytheon v. Roper*, 220 USPQ2d 592 (Fed. Cir. 1983); *Nestle v. Eugene*, 55 F.2d 854, 856, 12 USPQ 335 (6th Cir. 1932). Indeed, proof that the invention is made, used or sold by any person or entity other than the patentee is conclusive proof of utility. *United States Steel Corp.* v. *Phillips Petroleum Co.*, 865 F.2d 1247, 1252, 9 USPQ2d 1461 (Fed. Cir. 1989).

Over the past several years, a vibrant market has developed for databases containing all expressed genes (along with the polypeptide translations of those genes). (Note that the value in these databases is enhanced by their completeness, but each sequence in them is independently valuable.) The databases sold by Applicants' assignee, Incyte, include exactly the kinds of information made possible by the claimed invention, such as tissue and disease associations. Incyte sells its database containing the claimed sequence and millions of other sequences throughout the scientific community, including to pharmaceutical companies who use the information to develop new pharmaceuticals.

Both Incyte's customers and the scientific community have acknowledged that Incyte's databases have proven to be valuable in, for example, the identification and development of drug

candidates. As Incyte adds information to its databases, including the information that can be generated only as a result of Incyte's discovery of the claimed polypeptide, the databases become even more powerful tools. Thus the claimed invention adds more than incremental benefit to the drug discovery and development process.

III. The Patent Examiner's Rejections Are Without Merit

Rather than responding to the evidence demonstrating utility, the Examiner attempts to dismiss it altogether by arguing that the disclosed and well-established utilities for the claimed polypeptide are not "specific" or "well-established" utilities. (Office Action at p. 3.) The Examiner is incorrect both as a matter of law and as a matter of fact.

A. The Precise Biological Role Or Function Of An Expressed Polypeptide Is Not Required To Demonstrate Utility

The Patent Examiner's primary rejection of the claimed invention is based on the ground that, without information as to the precise biological role ("what IMP is, how it functions," as well as its "relationship to any specific disease;" Office Action at p. 3.) of the claimed invention, the claimed invention's utility is not sufficiently specific.

It may be that specific and substantial interpretations and detailed information on biological function are necessary to satisfy the requirements for publication in some technical journals, but they are not necessary to satisfy the requirements for obtaining a United States patent. The relevant question is not, as the Examiner would have it, whether it is known how or why the invention works, *In re Cortwright*, 165 F.3d 1353, 1359 (Fed. Cir. 1999), but rather whether the invention provides an "identifiable benefit" in presently available form. *Juicy Whip Inc.* v. *Orange Bang Inc.*, 185 F.3d 1364, 1366 (Fed. Cir. 1999). If the benefit exists, and there is a substantial likelihood the invention provides the benefit, it is useful. There can be no doubt, particularly in view of the Furness Declaration (at, *e.g.*, ¶¶ 10-13), that the present invention meets this test.

The threshold for determining whether an invention produces an identifiable benefit is low. *Juicy Whip*, 185 F.3d at 1366. Only those utilities that are so nebulous that a person of ordinary skill in the art would not know how to achieve an identifiable benefit and, at least according to the PTO guidelines, so-called "throwaway" utilities that are not directed to a person of ordinary skill in the art at all, do not meet the statutory requirement of utility. Utility Examination Guidelines, 66 Fed. Reg. 1092 (Jan. 5, 2001).

Knowledge of the biological function or role of a biological molecule has never been required to show real-world benefit. In its most recent explanation of its own utility guidelines, the PTO acknowledged as much (66 F.R. at 1095):

> [T]he utility of a claimed DNA does not necessarily depend on the function of the encoded gene product. A claimed DNA may have specific and substantial utility because, *e.g.*, it hybridizes near a disease-associated gene or it has gene-regulating activity.

By implicitly requiring knowledge of biological function for any claimed polypeptide, the Examiner has, contrary to law, elevated what is at most an evidentiary factor into an absolute requirement of utility. Rather than looking to the biological role or function of the claimed invention, the Examiner should have looked first to the benefits it is alleged to provide.

B. Membership in a Class of Useful Products Can Be Proof of Utility

Despite the uncontradicted evidence that the claimed polypeptide is a member of the integral membrane protein family, whose members indisputably are useful, the Examiner refused to impute the utility of the members of the integral membrane protein family to IMP. In the Office Action at p.6, the Patent Examiner takes the position that unless Applicants can identify which particular biological function within the class of integral membrane protein is possessed by IMP, utility cannot be imputed. To demonstrate utility by membership in the class of integral membrane proteins, the Examiner would require that all integral membrane proteins possess a "common" utility.

There is no such requirement in the law. In order to demonstrate utility by membership in a class, the law requires only that the class not contain a substantial number of useless members. So long

as the class does not contain a substantial number of useless members, there is sufficient likelihood that the claimed invention will have utility and a rejection under 35 U.S.C. § 101 is improper. That is true regardless of how the claimed invention ultimately is used and whether the members of the class possess one utility or many. *See Brenner* v. *Manson*, 383 U.S. 519, 532 (1966); *Application of Kirk*, 376 F.2d 936, 943 (CCPA 1967).

Membership in a "general" class is insufficient to demonstrate utility only if the class contains a substantial number of useless members. There would be, in that case, a substantial likelihood that the claimed invention is one of the useless members of the class. In the few cases in which class membership did not prove utility by substantial likelihood, the classes did in fact include predominately useless members. *E.g.*, *Brenner* (man-made steroids); *Kirk* (same); *Natta* (man-made polyethylene polymers).[1]

The Examiner addresses IMP as if the general class in which it is included is not the integral membrane protein family, but rather all polypeptides, including the vast majority of useless theoretical molecules not occurring in nature, and thus not pre-selected by nature to be useful. While these "general classes" may contain a substantial number of useless members, the integral membrane protein family does not. The integral membrane protein family is sufficiently specific to rule out any reasonable possibility that IMP would not also be useful like the other members of the family.

Because the Examiner has not presented any evidence that the integral membrane protein class of proteins has any, let alone a substantial number, of useless members, the Examiner must conclude that there is a "substantial likelihood" that the IMP encoded by the claimed polypeptide is useful.

Even if the Examiner's "common utility" criterion were correct – and it is not – the integral membrane protein family would meet it. It is undisputed that known members of the integral membrane protein family regulate membrane conductance and regulate ion channel activity. A person of ordinary skill in the art need not know any more about how the claimed invention regulates membrane

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[1]At a recent Biotechnology Customer Partnership Meeting, PTO Senior Examiner James Martinell described an analytical framework roughly consistent with this analysis. He stated that when an Applicants' claimed protein "is a member of a family of proteins that already are known based upon sequence homology," that can be an effective assertion of utility.

conductance to use it, and the Examiner presents no evidence to the contrary. Instead, the Examiner makes the conclusory observation that a person of ordinary skill in the art would need to know whether, for example, any given integral membrane protein regulates membrane conductance. The Examiner then goes on to assume that the only use for IMP absent knowledge as to how this member of the integral membrane protein family actually works is further study of IMP itself.

Not so. As demonstrated by Applicants, knowledge that IMP is an integral membrane protein is more than sufficient to make it useful for the diagnosis and treatment of various cancers. Indeed, IMP has been shown to be expressed in prostate, breast, and pancreatic tumor tissue libraries. The Examiner must accept these facts to be true unless the Examiner can provide evidence or sound scientific reasoning to the contrary. But the Examiner has not done so.

C. The uses of IMP in toxicology testing, drug discovery, and disease diagnosis are practical uses beyond mere study of the invention itself

To the extent that the Examiner rejected the claims at issue on the ground that the use of an invention as a tool for research is not a "substantial" use, the Examiner's rejection assumes a substantial overstatement of the law, and is incorrect in fact. Therefore, it must be overturned.

There is no authority for the proposition that use as a tool for research is not a substantial utility. Indeed, the Patent Office itself has recognized that just because an invention is used in a research setting does not mean that it lacks utility (Section 2107.01 of the Manual of Patent Examining Procedure, 8th Edition, August 2001, under the heading I. Specific and Substantial Requirements, Research Tools):

> Many research tools such as gas chromatographs, screening assays, and nucleotide sequencing techniques have a clear, specific and unquestionable utility (e.g., they are useful in analyzing compounds). An assessment that focuses on whether an invention is useful only in a research setting thus does not address whether the specific invention is in fact "useful" in a patent sense. Instead, Office personnel must distinguish between inventions that have a specifically identified substantial utility and inventions whose asserted utility requires further research to identify or reasonably confirm.

The PTO's actual practice has been, at least until the present, consistent with that approach. It has routinely issued patents for inventions whose only use is to facilitate research, such as DNA ligases, acknowledged by the PTO's Training Materials to be useful.

The subset of research uses that are not "substantial" utilities is limited. It consists only of those uses in which the claimed invention is to be an **object** of further study, thus merely inviting further research on the invention itself. This follows from *Brenner*, in which the U.S. Supreme Court held that a process for making a compound does not confer a substantial benefit where the only known use of the compound was to be the object of further research to determine its use. *Id.* at 535. Similarly, in *Kirk*, the Court held that a compound would not confer substantial benefit on the public merely because it might be used to synthesize some other, unknown compound that would confer substantial benefit. *Kirk*, 376 F.2d at 940, 945. ("What Applicants are really saying to those in the art is take these steroids, experiment, and find what use they do have as medicines.") Nowhere do those cases state or imply, however, that a material cannot be patentable if it has some other, additional beneficial use in research.

Such beneficial uses beyond studying the claimed invention itself have been demonstrated, in particular those described in the Furness Declaration. The Furness Declaration demonstrates that the claimed invention is a tool, rather than an object, of research, and it demonstrates exactly how that tool is used. Without the claimed invention, it would be more difficult to generate information regarding the properties of tissues, cells, drug candidates and toxins apart from additional information about the polypeptide itself.

D. The Patent Examiner Failed to Demonstrate That a Person of Ordinary Skill in the Art Would Reasonably Doubt the Utility of the Claimed Invention

Based principally on citations to scientific literature identifying some of the difficulties involved in predicting protein function, the Examiner rejected the pending claims on the ground that the applicant cannot impute utility to the claimed invention based on its structural similarity to another polypeptide undisputed by the Examiner to be useful. The Examiner's rejection is both incorrect as a matter of fact and as a matter of procedural law.

As demonstrated in § II.A., *supra*, the literature cited by the Examiner is not inconsistent with the Applicants' proof of homology by a reasonable probability. It may show that Applicants cannot prove function by homology with **certainty**, but Applicants need not meet such a rigorous standard of proof. Under the applicable law, once the applicant demonstrates a *prima facie* case of homology, the Examiner must accept the assertion of utility to be true unless the Examiner comes forward with evidence showing a person of ordinary skill would doubt the asserted utility could be achieved by a reasonable probability. *See In re Brana*, 51 F.3d at 1566; *In re Langer*, 503 F.2d 1380, 1391-92, 183 USPQ 288 (CCPA 1974). The Examiner has not made such a showing and, as such, the Examiner's rejection should be overturned.

In the present case, the Examiner contended that the degree of amino acid identity among IMP and other integral membrane family proteins is insufficient to establish that IMP is a member of the integral membrane family of proteins and thus shares the same utilities. The Examiner attempted to support this assertion with the teachings of Bowie et al. (Science (1990) 247:1306-1310), and Burgess et al. (J. Cell Biol. (1990) 111:2129-2138), all of record and addressed below. However, all of these references fail to support the outstanding rejections.

Applicants submit that the teachings of Bowie et al. are, in part, counter to the outstanding rejections, and in part, supportive of the asserted utilities of IMP based on amino acid sequence homology to integral membrane family proteins. Careful review of this reference reveals that the teachings of Bowie et al. are directed primarily toward studying the effects of site-directed substitution of amino acid residues in certain proteins in order to determine the relative importance of these residues to protein structure and function. As discussed below in further detail, such experiments are not relevant to Applicants' use of amino acid sequence homology to reasonably predict protein function.

In support of Applicants' use of amino acid sequence homology to reasonably predict the utility of the claimed polypeptide, Bowie et al. teach that evaluating sets of related sequences, which are members of the same gene family, is an accepted method of identifying functionally important residues that have been conserved over the course of evolution. (Bowie et al., page 1306, 1st column, last paragraph, and 2nd column, 2nd full paragraph; page 1308, 1st column, last paragraph; page 1310, 1st column, last paragraph.) It is known in the art that natural selection acts to conserve protein function.

As the Examiner stated and as taught by Bowie et al., proteins are tolerant of numerous amino acid substitutions that maintain protein function, and it is natural selection that permits these substitutions to occur. Conversely, mutations that reduce or abolish protein function are eliminated by natural selection. Based on these central tenets of molecular evolution, Applicants submit that the amino acid differences among Applicants' claimed polypeptide and known ion channel regulators are likely to occur at positions of minimal functional importance, while residues that are conserved are likely those that are important for protein function. One of ordinary skill in the art would further conclude that the level of conservation observed between Applicants' claimed polypeptide and ion channel regulators is indicative of a common function, and hence, common utility, among these proteins.

The Examiner further cited Burgess et al. as demonstrating the "sensitivity of proteins to alterations of even a single amino acid...". (Office Action at p. 5) However, these references are not relevant to the case at hand. Burgess et al. describe mutagenesis of HBGF-1 at an amino acid residue known to be important for ligand binding. In this cases, particular amino acid residues with known importance to protein function were specifically targeted for site-directed mutagenesis. These mutations were "artificially" created in the laboratory and, therefore, are **not** analogous to molecular evolution, which is profoundly influenced by natural selection. For example, the deactivating mutations as described by Burgess et al. would almost certainly not be tolerated in nature. Furthermore, it is clear that over the course of evolution, amino acid residues that are critical for protein function are **conserved**. Thus, the amino acid differences between SEQ ID NO:1 and stomatin and stomatin-like proteins are likely to represent substitutions that do **not** alter protein function. Therefore, the teachings of Burgess et al. are not relevant to the case at hand.

One could then argue that partial loss-of-function mutations do occur in nature, for example, the mutation in hemoglobin that causes sickle cell anemia. However, this example is the **rare** exception in evolution, **not the rule**. Persistence of such a mutation in a population would **not** be expected by one of ordinary skill in the art. Persistence occurs only because of the fluke of heterozygous advantage. Therefore, the Examiner's assertion that one of skill in the art would routinely expect to find single amino acid substitutions that drastically affect the function of the individual members of a conserved protein family is entirely unsubstantiated.

IV. By Requiring the Patent Applicant to Assert a Particular or Unique Utility, the Patent Examination Utility Guidelines and Training Materials Applied by the Patent Examiner Misstate the Law

There is an additional, independent reason to overturn the rejections: to the extent the rejections are based on Revised Interim Utility Examination Guidelines (64 FR 71427, December 21, 1999), the final Utility Examination Guidelines (66 FR 1092, January 5, 2001) and/or the Revised Interim Utility Guidelines Training Materials (USPTO Website www.uspto.gov, March 1, 2000), the Guidelines and Training Materials are themselves inconsistent with the law.

The Training Materials, which direct the Examiners regarding how to apply the Utility Guidelines, address the issue of specificity with reference to two kinds of asserted utilities: "specific" utilities, which meet the statutory requirements, and "general" utilities, which do not. The Training Materials define a "specific utility" as follows:

> A [specific utility] is *specific* to the subject matter claimed. This contrasts to *general* utility that would be applicable to the broad class of invention. For example, a claim to a polynucleotide whose use is disclosed simply as "gene probe" or "chromosome marker" would not be considered to be specific in the absence of a disclosure of a specific DNA target. Similarly, a general statement of diagnostic utility, such as diagnosing an unspecified disease, would ordinarily be insufficient absent a disclosure of what condition can be diagnosed.

The Training Materials distinguish between "specific" and "general" utilities by assessing whether the asserted utility is sufficiently "particular," *i.e.*, unique (Training Materials at p.52) as compared to the "broad class of invention." (In this regard, the Training Materials appear to parallel the view set forth in Stephen G. Kunin, Written Description Guidelines and Utility Guidelines, 82 J.P.T.O.S. 77, 97 (Feb. 2000) ("With regard to the issue of specific utility the question to ask is whether or not a utility set forth in the specification is *particular* to the claimed invention.").)

Such "unique" or "particular" utilities never have been required by the law. To meet the utility requirement, the invention need only be "practically useful," *Natta*, 480 F.2d 1 at 1397, and confer a "specific benefit" on the public. *Brenner*, 383 U.S. at 534. Thus incredible "throwaway" utilities, such as trying to "patent a transgenic mouse by saying it makes great snake food," do not meet this standard.

Karen Hall, Genomic Warfare, The American Lawyer 68 (June 2000) (quoting John Doll, Chief of the Biotech Section of USPTO).

This does not preclude, however, a general utility, contrary to the statement in the Training Materials where "specific utility" is defined (page 5). Practical real-world uses are not limited to uses that are unique to an invention. The law requires that the practical utility be "definite," not particular. *Montedison,* 664 F.2d at 375. Applicants are not aware of any court that has rejected an assertion of utility on the grounds that it is not "particular" or "unique" to the specific invention. Where courts have found utility to be too "general," it has been in those cases in which the asserted utility in the patent disclosure was not a practical use that conferred a specific benefit. That is, a person of ordinary skill in the art would have been left to guess as to how to benefit at all from the invention. In *Kirk*, for example, the CCPA held the assertion that a man-made steroid had "useful biological activity" was insufficient where there was no information in the specification as to how that biological activity could be practically used. *Kirk*, 376 F.2d at 941.

The fact that an invention can have a particular use does not provide a basis for requiring a particular use. *See Brana, supra* (disclosure describing a claimed antitumor compound as being homologous to an antitumor compound having activity against a "particular" type of cancer was determined to satisfy the specificity requirement). "Particularity" is not and never has been the *sine qua non* of utility; it is, at most, one of many factors to be considered.

As described *supra*, broad classes of inventions can satisfy the utility requirement so long as a person of ordinary skill in the art would understand how to achieve a practical benefit from knowledge of the class. Only classes that encompass a significant portion of nonuseful members would fail to meet the utility requirement. *Supra* § III.B. (*Montedison,* 664 F.2d at 374-75).

The Training Materials fail to distinguish between broad classes that convey information of practical utility and those that do not, lumping all of them into the latter, unpatentable category of "general" utilities. As a result, the Training Materials paint with too broad a brush. Rigorously applied, they would render unpatentable whole categories of inventions heretofore considered to be patentable, and that have indisputably benefitted the public, including the claimed invention. *See supra* § III.B. Thus the Training Materials cannot be applied consistently with the law.

V. Summary of Arguments Regarding Utility Rejection

Applicants respectfully submit that rejections for lack of utility based, *inter alia*, on an allegation of lack of specificity as set forth in the Office Action and as justified in the Revised Interim and final Utility Guidelines and Training Materials, are not supported in the law. Neither are they scientifically correct, nor supported by any evidence or sound scientific reasoning. These rejections are alleged to be founded on facts in court cases such as *Brenner* and *Kirk*, yet those facts are clearly distinguishable from the facts of the instant application, and indeed most if not all nucleotide and protein sequence applications. Nevertheless, the PTO is attempting to mold the facts and holdings of these prior cases, "like a nose of wax,"[2] to target rejections of claims to polypeptide and polynucleotide sequences, as well as to claims to methods of detecting said polynucleotide sequences, where biological activity information has not been proven by laboratory experimentation, and they have done so by ignoring perfectly acceptable utilities fully disclosed in the specifications as well as well-established utilities known to those of skill in the art. As is disclosed in the specification, and even more clearly, as one of ordinary skill in the art would understand, the claimed invention has well-established, specific, substantial and credible utilities. The rejections are, therefore, improper and should be withdrawn.

Moreover, to the extent the above rejections were based on the Revised Interim and final Examination Guidelines and Training Materials, those portions of the Guidelines and Training Materials that form the basis for the rejections should be determined to be inconsistent with the law.

Written description rejections under 35 U.S.C. § 112, first paragraph

Claims 1, 2, 17, and 18 have been rejected under the first paragraph of 35 U.S.C. 112 for alleged lack of an adequate written description. This rejection is respectfully traversed.

The requirements necessary to fulfill the written description requirement of 35 U.S.C. 112, first paragraph, are well established by case law.

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[2]"The concept of patentable subject matter under §101 is not 'like a nose of wax which may be turned and twisted in any direction * * *.' *White v. Dunbar*, 119 U.S. 47, 51." *(Parker v. Flook,* 198 USPQ 193 (US SupCt 1978))

> . . . the applicant must also convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession *of the invention.* The invention is, for purposes of the "written description" inquiry, *whatever is now claimed. Vas-Cath, Inc. v. Mahurkar*, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991)

Attention is also drawn to the Patent and Trademark Office's own "Guidelines for Examination of Patent Applications Under the 35 U.S.C. Sec. 112, para. 1", published January 5, 2001, which provide that :

> An applicant may also show that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics[42] which provide evidence that applicant was in possession of the claimed invention,[43] i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.[44] What is conventional or well known to one of ordinary skill in the art need not be disclosed in detail.[45] If a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification, then the adequate description requirement is met.[46] [footnotes omitted]

Thus, the written description standard is fulfilled by both what is specifically disclosed and what is conventional or well known to one skilled in the art.

SEQ ID NO:1 is specifically disclosed in the application (see, for example, pages 14-15, lines 7-11). Variants of SEQ ID NO:1 are described, for example, at page 15, lines 17-20. In particular, the preferred, more preferred, and most preferred IMP variants (80%, 90%, and 95% amino acid sequence similarity to SEQ ID NO:1) are described, for example, at page 15, lines 17-20. Incyte clones in which the nucleic acids encoding the human IMP were first identified and libraries from which those clones were isolated are described, for example, at page 14, line 1 of the Specification. Chemical and structural features of SEQ ID NO:1 are described, for example, on page 14, lines 7-18. SEQ ID NO:1, one of ordinary skill in the art would recognize naturally-occurring variants of SEQ ID NO:1 having 90% sequence identity to SEQ ID NO:1. Fragments of IMP could be made using either recombinant methods (e.g., see pages 19-24) or by chemical synthesis (e.g., see page 17, lines 15-22, and page 24, lines 13-19). "Biologically-active" fragments of IMP are defined at page 8, line 16-17. Methods for determining biological activity of IMP and fragments thereof are provided, e.g., at page 51, line 22-26. The Specification at pages 14-15 also discusses the biological activity of protein

homologs of IMP. Accordingly, the Specification provides an adequate written description of the recited polypeptide sequences.

A. The Specification provides an adequate written description of the claimed "variants" of SEQ ID NO:1.

The Office Action has further asserted that the claims are not supported by an adequate written description because

> "[t]he written description in this case only sets forth SEQ ID NO:1 and therefore the written description is not commensurate in scope with the claims which read on variants which as claimed, include naturally occurring polypeptides that are at least 90% identical to SEQ ID NO:1, and biologically active and immunogenic fragments of SEQ ID NO:1." Office Action at Page 8, lines 5-9.

Such a position is believed to present a misapplication of the law.

1. The present claims specifically define the claimed genus through the recitation of chemical structure

Court cases in which "DNA claims" have been at issue (which are hence relevant to claims to proteins encoded by the DNA and antibodies which specifically bind to the proteins) commonly emphasize that the recitation of structural features or chemical or physical properties are important factors to consider in a written description analysis of such claims. For example, in *Fiers v. Revel*, 25 USPQ2d 1601, 1606 (Fed. Cir. 1993), the court stated that:

> If a conception of a DNA requires a precise definition, such as by structure, formula, chemical name or physical properties, as we have held, then a description also requires that degree of specificity.

In a number of instances in which claims to DNA have been found invalid, the courts have noted that the claims attempted to define the claimed DNA in terms of functional characteristics without any reference to structural features. As set forth by the court in *University of California v. Eli Lilly and Co.*, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997):

> In claims to genetic material, however, a generic statement such as "vertebrate insulin cDNA" or "mammalian insulin cDNA," without more, is not an adequate written

description of the genus because it does not distinguish the claimed genus from others, except by function.

Thus, the mere recitation of functional characteristics of a DNA, without the definition of structural features, has been a common basis by which courts have found invalid claims to DNA. For example, in *Lilly*, 43 USPQ2d at 1407, the court found invalid for violation of the written description requirement the following claim of U.S. Patent No. 4,652,525:

> 1. A recombinant plasmid replicable in procaryotic host containing within its nucleotide sequence a subsequence having the structure of the reverse transcript of an mRNA of a vertebrate, which mRNA encodes insulin.

In *Fiers*, 25 USPQ2d at 1603, the parties were in an interference involving the following count:

> A DNA which consists essentially of a DNA which codes for a human fibroblast interferon-beta polypeptide.

Party Revel in the *Fiers* case argued that its foreign priority application contained an adequate written description of the DNA of the count because that application mentioned a potential method for isolating the DNA. The Revel priority application, however, did not have a description of any particular DNA structure corresponding to the DNA of the count. The court therefore found that the Revel priority application lacked an adequate written description of the subject matter of the count.

Thus, in *Lilly* and *Fiers*, nucleic acids were defined on the basis of functional characteristics and were found not to comply with the written description requirement of 35 U.S.C. §112; *i.e.*, "an mRNA of a vertebrate, which mRNA encodes insulin" in *Lilly*, and "DNA which codes for a human fibroblast interferon-beta polypeptide" in *Fiers*. In contrast to the situation in *Lilly* and *Fiers*, the claims at issue in the present application define polypeptides in terms of chemical structure, rather than on functional characteristics. For example, the "variant language" of independent claim 1 recites chemical structure to define the claimed genus:

> 2. An isolated and purified polynucleotide sequence encoding a polypeptide selected from the group consisting of:...b) a naturally-occurring amino acid sequence having at least 90% sequence identity to the sequence of SEQ ID NO:1...

From the above it should be apparent that the claims of the subject application are fundamentally different from those found invalid in *Lilly* and *Fiers*. The subject matter of the present claims is defined in terms of the chemical structure of SEQ ID NO:1. In the present case, there is no reliance merely on a description of functional characteristics of the polypeptides recited by the claims. In fact, there is no recitation of functional characteristics. Moreover, if such functional recitations were included, it would add to the structural characterization of the recited polypeptides. The polypeptides defined in the claims of the present application recite structural features, and cases such as *Lilly* and *Fiers* stress that the recitation of structure is an important factor to consider in a written description analysis of claims of this type. By failing to base its written description inquiry "on whatever is now claimed," the Office Action failed to provide an appropriate analysis of the present claims and how they differ from those found not to satisfy the written description requirement in *Lilly* and *Fiers*.

2. The state of the art at the time of the present invention is further advanced than at the time of the *Lilly* and *Fiers* applications

In the *Lilly* case, claims of U.S. Patent No. 4,652,525 were found invalid for failing to comply with the written description requirement of 35 U.S.C. §112. The '525 patent claimed the benefit of priority of two applications, Application Serial No. 801,343 filed May 27, 1977, and Application Serial No. 805,023 filed June 9, 1977. In the *Fiers* case, party Revel claimed the benefit of priority of an Israeli application filed on November 21, 1979. Thus, the written description inquiry in those case was based on the state of the art at essentially at the "dark ages" of recombinant DNA technology.

The present application has a priority date of January 9, 1997. Much has happened in the development of recombinant DNA technology in the 17 or more years from the time of filing of the applications involved in *Lilly* and *Fiers* and the present application. For example, the technique of polymerase chain reaction (PCR) was invented. Highly efficient cloning and DNA sequencing technology has been developed. Large databases of protein and nucleotide sequences have been compiled. Much of the raw material of the human and other genomes has been sequenced. With these remarkable advances one of skill in the art would recognize that, given the sequence information of SEQ ID NO:1, and the additional extensive detail provided by the subject application, the present

inventors were in possession of the claimed polynucleotide variants at the time of filing of this application.

3. Summary

The Office Action failed to base its written description inquiry "on whatever is now claimed." Consequently, the Action did not provide an appropriate analysis of the present claims and how they differ from those found not to satisfy the written description requirement in cases such as *Lilly* and *Fiers*. In particular, the claims of the subject application are fundamentally different from those found invalid in *Lilly* and *Fiers*. The subject matter of the present claims is defined in terms of the chemical structure of SEQ ID NO:1. The courts have stressed that structural features are important factors to consider in a written description analysis of claims to nucleic acids and proteins. In addition, the genus of polypeptides defined by the present claims is adequately described, as evidenced by Brenner et al. and consideration of the claims of the '740 patent involved in *Lilly*. Furthermore, there have been remarkable advances in the state of the art since the *Lilly* and *Fiers* cases, and these advances were given no consideration whatsoever in the position set forth by the Office Action.

Prior Art Rejections – 35 U.S.C. § 102

I. Rejection of Claims 1 and 17 Under 35 U.S.C. § 102(b)

Claims 1 and 17 have been rejected under 35 U.S.C. §102(b) as being anticipated by Wakefield et al. (U.S. Patent No. 5,534,619). To support this rejection, the Examiner has provided a sequence alignment showing that a 7 amino acid stretch of a sequence disclosed in Wakefield et al. is identical to a 7 amino acid stretch of the sequence set forth in Applicants' SEQ ID NO: 1. Claims 1 and 17 are drawn, in part, to a polypeptide comprising an immunogenic fragment of a polypeptide having an amino acid sequence of SEQ ID NO: 1, and a composition comprising the composition of claim 1 and a pharmaceutically acceptable excipient, respectively.

Wakefield et al. is directed to heparin anticoagulation proteins and polynucleotides encoding those proteins. There is no apparent relationship between the function of the Wakefield proteins and the integral membrane protein of the present application. Nevertheless, the Office Action has made the unsubstantiated assertion that the protein fragment disclosed in Wakefield proteins would be immunogenic. There is no evidence that the amino acid sequence of Wakefield which is homologous to a seven amino acid stretch of Applicants' polypeptide is immunogenic.

Regardless, in the interest of expediting prosecution of the subject application, claim 1 has been revised to recite, *inter alia*, an immunogenic fragment of a polypeptide having an amino acid sequence of SEQ ID NO:1, said fragment comprising at least 15 contiguous amino acid residues. Wakefield et al. does not have any disclosure specifying such fragments or antibodies which would bind thereto. By this amendment, Applicants expressly do not disclaim equivalents of the invention which could include polynucleotides encoding immunogenic fragments comprising fewer than 15 contiguous amino acid residues of SEQ ID NO:1. Applicants do not concede to the Patent Office position; Applicants are amending the claims solely to obtain expeditious allowance of the instant application. While not conceding to the Patent Office position, it is believed that claim 1, as amended, and dependent claim 7, recite patentable subject matter. Therefore, withdrawal of this rejection is requested.

II. Rejection of Claims 1 and 17 Under 35 U.S.C. § 102(e)

Claims 1 and 17 have also been rejected under 35 U.S.C. § 102(e) as being anticipated by Zhang et al. (U.S. Patent No. 5,670,483). To support this rejection, the Examiner has provided a sequence alignment showing that a 7 amino acid stretch of a sequence disclosed in Zhang et al. is identical to a 7 amino acid stretch of the sequence set forth in Applicants' SEQ ID NO: 1. The Office Action asserts that "[b]ecause any fragment is potentially immunogenic given the correct dosage and concentration, the protein fragment disclosed by Zhang et al [*sic*] in the absence of evidence to the contrary would be immunogenic" (Office Action at p. 11). This rejection is traversed.

Zhang et al. is directed to a macroscopic membrane formed by amphiphilic peptides. There is no apparent relationship between the function of the Zhang proteins and the integral membrane protein of the present application. Nevertheless, the Office Action has made the unsubstantiated assertion that the polypeptide sequence disclosed in Zhang would encode an immunogenic polypeptide. There is no evidence that the amino acid sequence of Zhang which is homologous to a seven amino acid stretch of Applicants' polypeptide is immunogenic.

To expedite prosecution, claim 1 has been amended to recite, *inter alia*, an immunogenic fragment of a polypeptide having an amino acid sequence of SEQ ID NO:1, said fragment comprising at least 15 contiguous amino acid residues. Support for this amendment can be found in the specification at, for example, page 51, line 1. By this amendment, Applicants expressly do not disclaim equivalents of the invention which could include polynucleotides encoding immunogenic fragments comprising fewer than 15 contiguous amino acid residues of SEQ ID NO:1. Applicants do not

concede to the Patent Office position; Applicants are amending the claims solely to obtain expeditious allowance of the instant application. While not conceding to the Patent Office position, it is believed that claim 1, as amended, and dependent claim 7, recite patentable subject matter. Therefore, withdrawal of this rejection is requested.

CONCLUSION

In light of the above amendments and remarks, Applicants submit that the present application is fully in condition for allowance, and request that the Examiner withdraw the outstanding rejections. Early notice to that effect is earnestly solicited.

If the Examiner contemplates other action, or if a telephone conference would expedite allowance of the claims, Applicants invite the Examiner to contact Applicants' Attorney at (650) 855-0555.

Applicants believe that no fee is due with this communication. However, if the USPTO determines that a fee is due, the Commissioner is hereby authorized to charge Deposit Account No. **09-0108.**

Respectfully submitted,
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VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE CLAIMS:

1. (Once amended) An isolated polypeptide comprising an amino acid sequence selected from the group consisting of:

a) a polypeptide comprising an amino acid sequence of SEQ ID NO:1,

b) a naturally occurring polypeptide comprising an amino acid sequence at least 90% identical to an amino acid sequence of SEQ ID NO:1,

c) a biologically active fragment of a polypeptide having an amino acid sequence of SEQ ID NO:1, and

d) an immunogenic fragment of a polypeptide having an amino acid sequence of SEQ ID NO:1, said fragment comprising at least 15 contiguous amino acid residues.

2

Progress with Proteome Projects: Why all Proteins Expressed by a Genome Should be Identified and How To Do It

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Introduction

The advent of large genome sequencing projects has changed the scale of biology. Over a relatively short period of time, we have witnessed the elucidation of the complete nucleotide sequence for bacteriophage λ (Sanger *et al.*, 1982), the nucleotide sequence of an eukaryotic chromosome (Oliver *et al.*, 1992), and in the near future will see the definition of all open reading frames of some simple organisms, including *Mycoplasma pneumoniae*, *Escherichia coli*, *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Arabidopsis thaliana*. Nevertheless, genome sequencing projects are not an end in themselves. In fact, they only represent a starting point to understanding the function of an organism. A great challenge that biologists now face is how the co-expression of thousands of genes can best be examined under physiological and pathophysiological conditions, and how these patterns of expression define an organism.

There are two approaches that can be used to examine gene expression on a large scale. One uses nucleic acid-based technology, the other protein-based technology. The most promising nucleic-acid based technology is differential display of mRNA (Liang and Pardee, 1992; Bauer *et al.*, 1993), which uses polymerase chain reaction with arbitrary primers to generate thousands of cDNA species, each which correspond to an expressed gene or part of a gene. However, it is currently unclear if this technique can be developed to reliably assay the expression of thousands of genes or

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identify all cDNA species, and the approach does not easily allow a systematic screening. Analysis of gene expression by the study of proteins present in a cell or tissue presents a favorable alternative. This can be achieved by use of two-dimensional (2-D) gel electrophoresis, quantitative computer image analysis, and protein identification techniques to create 'reference maps' of all detectable proteins. Such reference maps establish patterns of normal and abnormal gene expression in the organism, and allow the examination of some post-translational protein modifications which are functionally important for many proteins. It is possible to screen proteins systematically from reference maps to establish their identities.

To define protein-based gene expression analysis, the concept of the 'proteome' was recently proposed (Wilkins *et al.*, 1995; Wasinger *et al.*, 1995). A proteome is the entire PROTein complement expressed by a genOME, or by a cell or tissue type. The concept of the proteome has some differences from that of the genome, as while there is only one definitive genome of an organism, the proteome is an entity which can change under different conditions, and can be dissimilar in different tissues of a single organism. A proteome nevertheless remains a direct product of a genome. Interestingly, the number of proteins in a proteome can exceed the number of genes present, as protein products expressed by alternative gene splicing or with different post-translational modifications are observed as separate molecules on a 2-D gel. As an extrapolation of the concept of the 'genome project', a 'proteome project' is research which seeks to identify and characterise the proteins present in a cell or tissue and define their patterns of expression.

Proteome projects present challenges of a similar magnitude to that of genome projects. Technically, the 2-D gel electrophoresis must be reproducible and of high resolution, allowing the separation and detection of the thousands of proteins in a cell. Low copy number proteins should be detectable. There should be computer gel image analysis systems that can qualitatively and quantitatively catalog the electrophoretically separated proteins, to form reference maps. A range of rapid and reliable techniques must be available for the identification and characterisation of proteins. As a consequence of a proteome project, protein databases must be assembled that contain reference information about proteins; such databases must be linked to genomic databases and protein reference maps. Databases should be widely accessible and easy to use.

Recently, there have been many changes in the techniques and resources available for the analysis of proteomes. It is the aim of this chapter to discuss the status of the areas outlined above, and to review briefly the progress of some current proteome projects.

Two-dimensional electrophoresis of proteomes

Two dimensional (2-D) gel electrophoresis involves the separation of proteins by their isoelectric point in the first dimension, then separation according to molecular weight by sodium dodecyl sulfate electrophoresis in the second dimension. Since first described (Klose, 1975; O'Farrell, 1975; Scheele, 1975), it has become the method of choice for the separation of complex mixtures of proteins, albeit with many modifications to the original techniques. 2-D electrophoresis forms the basis of proteome projects through separating proteins by their size and charge (Hochstrasser *et al.*,

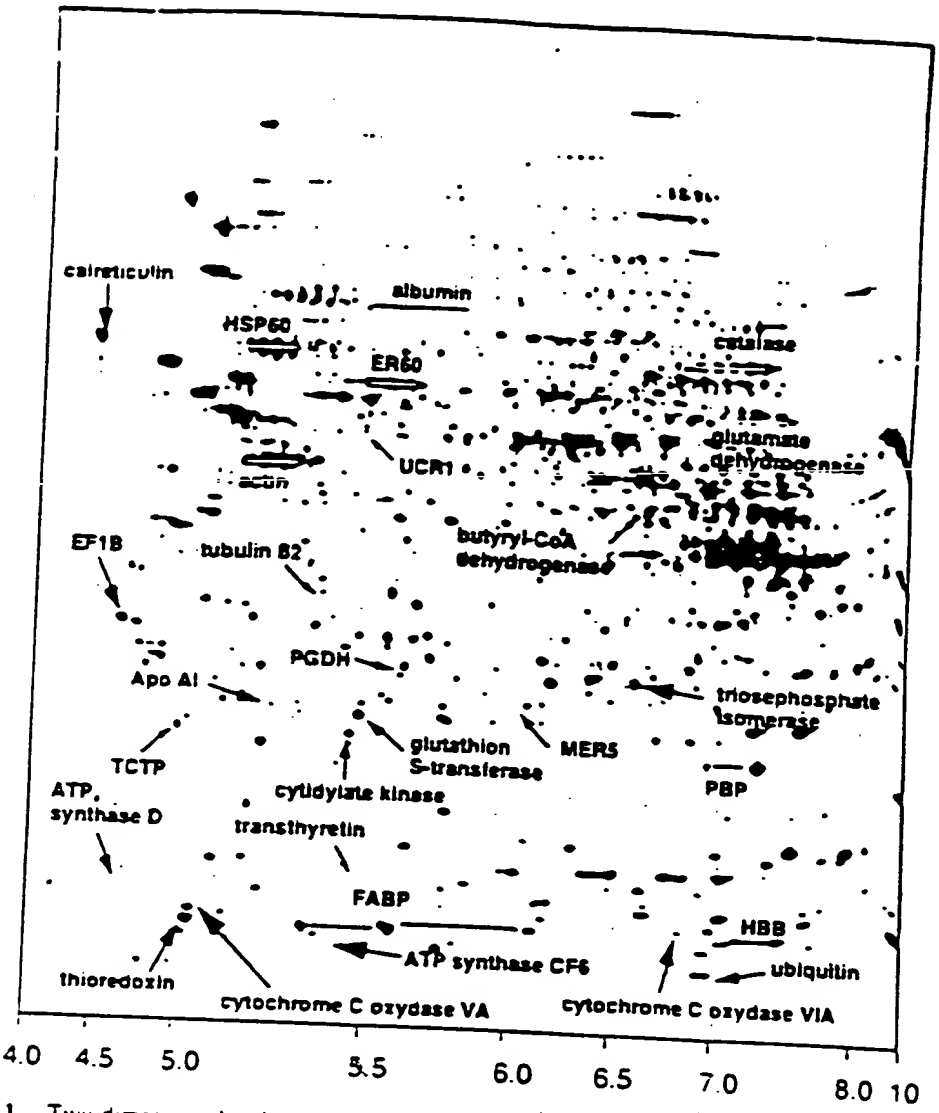

Figure 1. Two-dimensional gel electrophoresis map of a human hepatoblastoma-derived cell line, illustrating the very high resolution of the technique. The first dimensional separation (right to left of figure) was achieved using immobilised pH gradient electrophoresis of 4.0 to 10.0 units. The second dimension (top to bottom of figure) was SDS-PAGE using a 11%–14% acrylamide gradient, allowing separation in the molecular weight range 10–250 kDa. Proteins were visualised by silver staining. Arrows show proteins of known identity.

1992; Celis *et al.*, 1993; Garrels and Franza, 1989; VanBogelen *et al.*, 1992). Current protocols can resolve two to three thousand proteins from a complex sample on a single gel (*Figure 1*).

2-D GEL RESOLUTION AND REPRODUCIBILITY

A primary challenge of separating complex mixtures of proteins by 2-D gel electrophoresis has been to achieve high resolution and reproducibility. High resolution ensures that a maximum of protein species are separated, and high reproducibility is

vital to allow comparison of gels from day to day and between research sites. These factors can be difficult to achieve.

Carrier ampholytes are a common means of isoelectric focusing for the first dimension of 2-D electrophoresis. Gels are usually focused to equilibrium to separate proteins in the pI range 4 to 8, and run in a non-equilibrium mode (NEPHGE) to separate proteins of higher pI (7 to 11.5) (O'Farrell, 1975; O'Farrell, Goodman and O'Farrell, 1977). Unfortunately, the use of carrier ampholytes in the isoelectric focusing procedure is susceptible to 'cathode drift', whereby pH gradients established by prefocusing of ampholytes slowly change with time (Righetti and Drysdale, 1983). Carrier ampholyte pH gradients are also distorted by high salt concentration of samples (Bjellqvist *et al.*, 1982), and by high protein load (O'Farrell, 1975). A further limitation is that iso electric focusing gels, which are cast and subject to electrophoresis in narrow glass tubes, need to be extruded by mechanical means before application to the second dimension – a procedure that potentially distorts the gel. Nevertheless, many of the above shortcomings can be avoided by loading small amounts of ^{14}C or ^{35}S radiolabelled samples (Garrels, 1989; Neidhardt *et al.*, 1989; Vandekerkhove *et al.*, 1990). High sensitivity detection is then achieved through use of fluorography or phosphorimaging plates (Bonner and Laskey, 1974; Johnston, Pickett and Barker, 1990; Patterson and Latter, 1993). However, this approach is only practicable for organisms or tissues that can be radiolabelled.

An alternative technique, which is becoming the method of choice for the first dimension separation of proteins, involves isoelectric focusing in immobilized pH gradient (IPG) gels (Bjellqvist *et al.*, 1982; Görg, Postel and Gunther, 1988; Righetti, 1990). Immobilized pH gradients are formed by the covalent coupling of the pH gradient into an acrylamide matrix, creating a gradient that is completely stable with time. IPG gels are usually poured onto a stiff backing film, which is mechanically strong and provides easy gel handling (Ostergren, Eriksson and Bjellqvist, 1988). The major advantages of IPG separations are that they do not suffer from cathodic drift, they allow focusing of basic and very acidic proteins to equilibrium, pH gradients can be precisely tailored (linear, stepwise, sigmoidal), and that separations over a very narrow pH range are possible (0.05 pH units per cm) (Righetti, 1990; Bjellqvist *et al.*, 1982, 1993a; Sinha *et al.*, 1990; Görg *et al.*, 1988; Gelfi *et al.*, 1987; Gunther *et al.*, 1988). However, it is not currently possible to use IPG gels to separate very basic proteins of isoelectric point greater than 10, although this is under development. Narrow pH range separations are useful to address problems of protein co-migration in complex samples, allowing 'zooming in' on regions of a gel (*Figure 2*). IPG gel strips are now commercially available, which begin to address the problems of intra- and inter-lab isoelectric focusing reproducibility.

There are two means of electrophoresis for the second dimension separation of proteins: vertical slab gels and horizontal ultrathin gels (Görg, Postel, and Gunther, 1988). Both are usually SDS-containing gradient gels of approximately 11% to 15% acrylamide, which separate proteins in the molecular mass range of 10 – 150kD. A stacking gel is not usually used with slab gels, but is necessary when using horizontal gel setups (Görg, Postel and Gunther, 1988). Comparisons have shown that there is little or no difference in the reproducibility of electrophoresis using either approach (Corbett *et al.*, 1994a), but commercially available vertical or horizontal precast gels will provide greater reproducibility for occasional users. For slab gel electrophoresis,

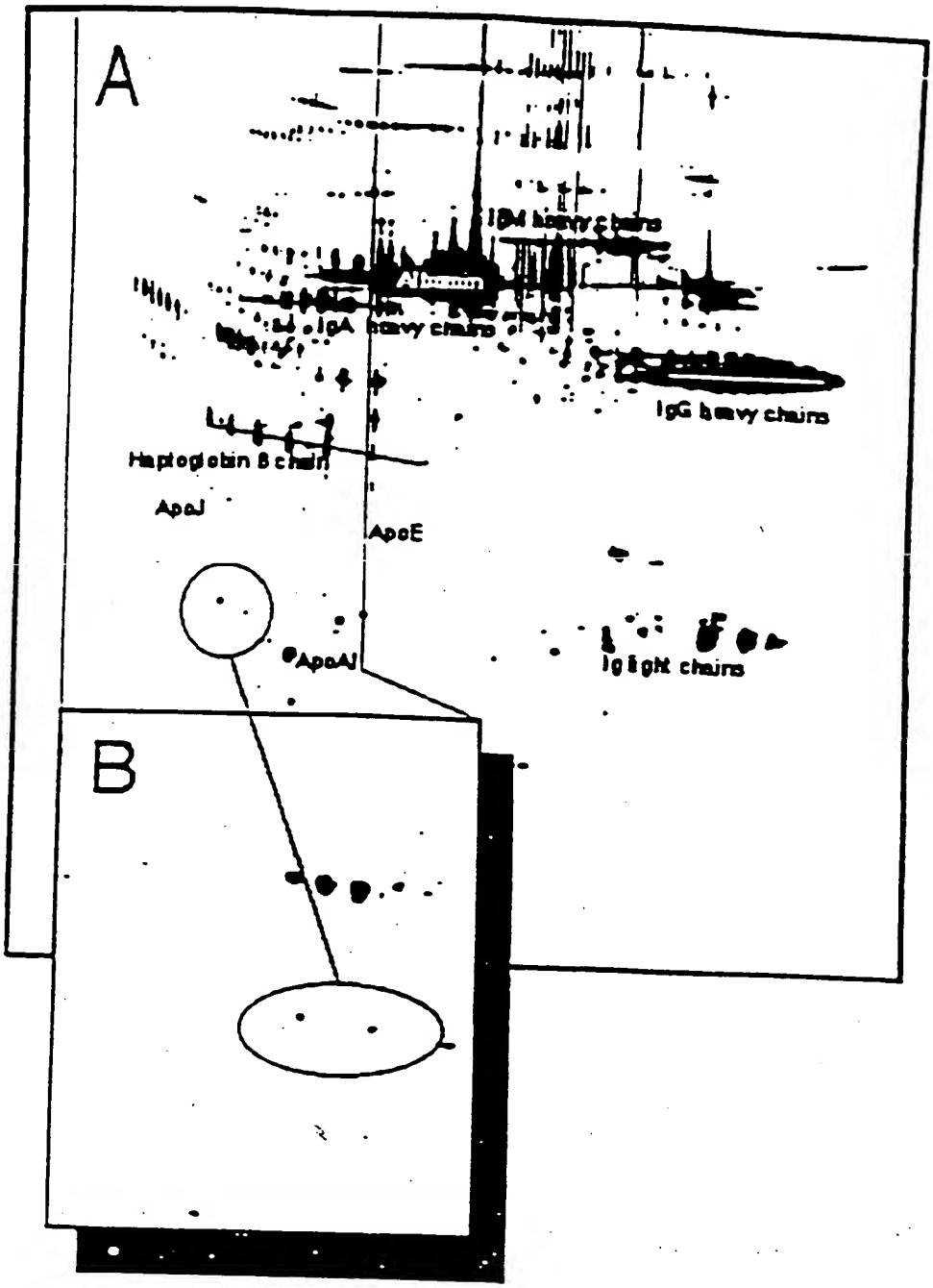

Figure 2. Two-dimensional gel electrophoresis allows 'zooming in' on areas of interest. Rings highlight 2 proteins common to each gel. (A) Wide pI range two dimensional electrophoresis map of human plasma proteins. First dimension separation was achieved using an immobilised pH gradient of 3.5 to 10.0 units. The second dimension was SDS-PAGE. Actual gel size was 16cm × 20cm, and proteins were visualised with silver staining. (B) Narrow pI range electrophoresis was used to 'zoom in' on a small region of the plasma map. The first dimension used a narrow range immobilised pH gradient of 4.2 to 5.2 units, and second dimension was SDS-PAGE. Micropreparative loading was used, and the gel blotted to PVDF. Proteins were visualised with amido black. Actual blot size was 16cm × 20cm.

the use of piperazine diacrylyl as a gel crosslinker and the addition of thiosulfate in the catalyst system has been shown to give better resolution and higher sensitivity detection (Hochstrasser and Merril, 1988; Hochstrasser, Patchornik and Merril, 1988).

Notwithstanding the advances described above, there is an increasing demand to improve the reproducibility of 2-D electrophoresis to facilitate database construction and proteome studies. Harrington *et al.* (1993) explain that if a gel resolves 4000 protein spots, and there is 99.5% spot matching from gel to gel, this will produce 20 spot errors per gel. This amount of error, which might accumulate with each gel to gel comparison used in database construction, could produce an unacceptable degree of uncertainty in gel databases. To address these issues, partial automation of large 2-D gel separations has been undertaken (Nokihara, Morita and Kuriki, 1992; Harrington *et al.*, 1993). Although results are preliminary, spot to spot positional reproducibility in one study was found to be threefold improved over manual methods (Harrington *et al.*, 1993). It should be noted that small 2-D gel formats (50 × 43 mm) have been almost completely automated (Brewer *et al.*, 1986), although these are not generally used for database studies.

MICROPREPARATIVE 2-D GEL ELECTROPHORESIS

With the advent of affordable protein microcharacterisation techniques, including N-terminal microsequencing, amino acid analysis, peptide mass fingerprinting, phosphate analysis and monosaccharide compositional analysis, a new challenge for 2-D electrophoresis has been to maintain high resolution and reproducibility but to provide protein in sufficient quantities for chemical analysis (high nanogram to low microgram quantities of proteins per spot). This becomes difficult to achieve with very complex samples such as whole bacterial cells, as the initial protein load is divided among 2000 to 4000 protein species. Two approaches are used for producing amounts of material that can be chemically characterised. The first method is to run multiple gels, collect and pool the spots of interest, and subject them to concentration (Ji *et al.*, 1994; Walsh *et al.*, 1995; Rasmussen *et al.*, 1992). In this approach, the concentration process must also act as a purification step to remove accumulated electrophoretic contaminants such as glycine. A more elegant approach has been to exploit the high loading capacity of IPG isoelectric focusing. The high loading capacity of immobilised pH gradients was described early (Ek, Bjellqvist and Righetti, 1983), but has only recently been applied to 2-D electrophoresis (Hanash *et al.*, 1991; Bjellqvist *et al.*, 1993b). Up to 15 mg of protein can been applied to a single gel, yielding microgram quantities of hundreds of protein species. A further benefit of this approach is that proteins present in low abundance, which may not be visualised by lower protein loads, are more likely to be detected. The use of electrophoretic or chromatographic prefractionation techniques (Hochstrasser *et al.*, 1991a; Harrington *et al.*, 1992), followed by high loading of narrow-range IPG separations (Bjellqvist *et al.*, 1993b) provides a likely solution to studies on proteins present in low abundance.

Methods of protein detection

There are many means for detecting proteins from 2-D gels. The method used will be dictated by factors including protein load on gel (analytical or preparative), the purpose of the gel (for protein quantitation or for blotting and chemical characterisation), and the sensitivity required. The most common means of protein detection and their applications are shown in *Table 1*. Most detection methods have drawbacks, for

Table 1: Common stains for 2-D gels or blots and their applications.

Detection Method	Main applications	Unsuitable applications	Sensitivity	References
[^{35}S] Met or ^{14}C radiolabelling and fluorography or phosphorimaging	Cell lines, cultured organisms	Samples that cannot be labelled	20 ppm of radiolabel in a spot	Garels and Franza, 1986 Latham, Garrels and Solter, 1993
[^{35}S]thiourea silver	Extremely high sensitivity gel staining	Preparative 2-D, PVDF or NC membranes	0.3 ng protein on spot or band of gel	Wallace and Saluz, 1992a,b
Silver	Very high sensitivity gel staining, can be mono or polychromatic	Preparative 2-D, PVDF or NC membranes	4 ng protein on spot or band of gel	Rabilloud, 1992; Hochstrasser and Merril, 1988
Coomassie blue R-250	Staining of gels, staining of PVDF membranes before protein sequencing	Staining prior to direct mass determination from PVDF; amino acid analysis on PVDF; detection of some glycoproteins	40 ng protein on band or spot of gel	Strupat *et al.*, 1994; Gharahdaghi *et al.*, 1992; Goldberg *et al.*, 1988; Sanchez *et al.*, 1992
Colloidal gold	Staining NC membranes, staining PVDF before direct MALDI-TOF	Gels	60 × higher than coomassie	Yamaguchi and Asakawa, 1988; Eckerskorn *et al.*, 1992; Strupat *et al.*, 1994
Zinc imidazole	Reverse staining of gels or membranes; may be beneficial in MALDI-TOF of peptides	Where positive image is required	Higher than coomassie	Ortiz *et al.*, 1992; James *et al.*, 1993
Ponceau S and amido black	Staining higher protein loads on PVDF, for protein sequencing or amino acid analysis	Staining prior to direct mass determination from PVDF	100 ng protein on band or spot of gel	Sanchez *et al.*, 1992; Strupat *et al.*, 1994; Wilkins *et al.*, 1995
India ink	Staining of membrane-bound proteins, staining PVDF before direct MALDI-TOF	Gel staining, not quantitative from protein to protein	1–10 ng	Li *et al.*, 1989; Hughes, Mack and Hamparian, 1988; Strupat *et al.*, 1994
Stains-all	Staining to detect glycoproteins or Ca^{2+} binding proteins	General gel staining	100 ng protein on band or spot of gel	Campbell, MacLennan and Jorgensen, 1983; Goldberg *et al.*, 1988

PVDF = polyvinylidene difluoride, NC = nitrocellulose, MALDI-TOF = matrix assisted laser desorption ionisation time of flight mass spectrometry

example, some glycoproteins are not stained by coomassie blue (Goldberg *et al.*, 1988), and many organic dyes are unsuitable for protein detection on PVDF if samples are to be used for direct matrix-assited laser desorption ionisation mass spectrometry (Strupat *et al.*, 1994).

Although most means of protein detection give some indication of the quantities of protein present, in general they cannot be used for global quantitation. This is because

no protein stain is able consistently to detect proteins over a wide range of concentrations, isoelectric points and amino acid compositions, and with a variety of post-translational modifications (Goldberg *et al.*, 1988; Li *et al.*, 1989). Furthermore, there are large differences in staining pattern when identical gels or blots are subjected to different stains, including amido black, imidazole zinc, india ink, ponceau S, colloidal gold, or coomassie blue (Tovey, Ford and Baldo, 1987; Ortiz *et al.*, 1992). The most common means of quantitating large numbers of proteins in a 2-D gel involves the radiolabelling of protein samples prior to electrophoresis, and protein quantitation based on fluorography and image analysis or liquid scintillation counting (Garrels, 1989; Celis and Olsen, 1994). However, proteins which do not contain methionine cannot be detected if only [^{35}S] methionine is used for labelling. Amino acid analysis of protein spots visualised by other techniques presents a likely means of protein quantitation for the future.

BLOTTING OF PROTEINS TO MEMBRANES

Electrophoretic blotting of proteins from two-dimensional polyacrylamide gels to membranes presents many options for protein identification and microcharacterisation which are not possible when proteins remain in gels. For example, when proteins are blotted to polyvinylidene difluoride (PVDF) membranes, they can be identified by N-terminal sequencing, amino acid analysis, or immunoblotting, or they may be subjected to endoproteinase digestion, monosaccharide analysis, phosphate analysis, or direct matrix-assisted laser desorption ionisation mass spectrometry (Matsudaira, 1987; Wilkins *et al.*, 1995; Jungblut *et al.*, 1994; Sutton *et al.*, 1995; Rasmussen *et al.*, 1994; Weizthandler *et al.*, 1993; Murthy and Iqbal, 1991; Eckerskorn *et al.*, 1992). It is possible to combine of some of these procedures on a single protein spot on a PVDF membrane (Packer *et al.*, 1995; Wilkins *et al.*, submitted; Weizthandler *et al.*, 1993). This is useful when minimal amounts of protein are available for analysis. These techniques will be explored in detail later in this review. Notwithstanding the above, there are some disadvantages associated with blotting of proteins to membranes. There is always loss of sample during blotting procedures (Eckerskorn and Lottspeich, 1993), and common protein detection methods are less sensitive or not applicable to membranes (*Table 1*), presenting difficulties for the analysis of low abundance proteins. Detailed discussion of the merits of available membranes and common blotting techniques can be found elsewhere (Eckerskorn and Lottspeich, 1993; Strupat *et al.*, 1994; Patterson, 1994).

2-D gel analysis, documentation, and proteome databases

Following protein electrophoresis and detection, detailed analysis of gel images is undertaken with computer systems. For proteome projects, the aim of this analysis is to catalogue all spots from the 2-D gel in a qualitative and if possible quantitative manner, so as to define the number of proteins present and their levels of expression. Reference gel images, constructed from one or more gels, form the basis of two-dimensional gel databases. These databases also contain protein spot identities and

details of their post-translational modifications. 2-D gel databases are beginning to be linked to or integrated with comprehensive protein and nucleic acid databases (Neidhardt *et al.*, 1989; Simpson *et al.*, 1992; Appel *et al.*, 1994), and 'organism' databases, containing DNA sequence data, chromosomal map locations, reference 2-D gels and protein functional information for an organism, are becoming established as genome and proteome projects progress (VanBogelen *et al.*, 1992; Yeast Protein Database cited in Garrels *et al.*, 1994).

GEL IMAGE ANALYSIS AND REFERENCE GELS

After 2-D electrophoresis and protein visualisation by staining, fluorography or phosphorimaging, images of gels are digitised for computer analysis by an image scanner, laser densitomer, or charge-coupled device (CCD) camera (Garrels, 1989; Celis *et al.*, 1990a; Urwin and Jackson, 1993). All systems digitise gels with a resolution of 100 – 200 mm, and can detect a wide range of densities or shading (256 or more 'grey scales'). Following this, gel images are subjected to a series of manipulations to remove vertical and horizontal streaking and background haze, to detect spot positions and boundaries, and to calculate spot intensity (*Figure 3*). A standard spot (SSP) number, containing vertical and horizontal positional information, is assigned to each detected spot and becomes the protein's reference number. *Table 2* lists some notable software packages which process 2-D gel images.

Table 2: Some Software Packages for the Analysis of Gel Images.

Gel Image Analysis System	References*
ELSIE 3 & 5	Olsen and Miller, 1988; Wirth *et al.*, 1991; Wirth *et al.*, 1993
GELLAB I & II	Wu, Lemkin and Upton, 1993; Lemkin, Wu and Upton, 1993; Myrick *et al.*, 1993.
MELANIE I & II	Appel, *et al.* 1991; Hochstrasser *et al.* 1991b
QUEST I & II and PDQUEST	Garrels, 1989; Monardo *et al.*, 1994; Holt *et al.*, 1992; Celis *et al.*, 1990a,b
TYCHO & KEPLAR	Anderson *et al.*, 1984; Richardson, Horn and Anderson, 1994

*These references are not exhaustive, they include some references of use as well as authors of the system

As there are difficulties in the electrophoresis of samples with 100% reproducibility, reference gel images are often constructed from many gels of the same sample (Garrels and Franza, 1989; Neidhardt *et al.*, 1989). Since this involves the matching of 2000 to 4000 proteins from one gel to another, it presents a considerable challenge to image analysis systems. Matching of gels is usually initiated by an operator, who manually designates approximately 50 or so prominent spots as 'landmarks' on gels to be cross-matched. Proteins which match are then established around landmarks, using computer-based vector algorithms to extend the matching over the entire gel. Close to 100% of spots from complex samples can be matched by these methods, although different degrees of operator intervention may be required (Olsen and Miller, 1988; Lemkin and Lester, 1989; Garrels, 1989; Myrick *et al.*, 1993).

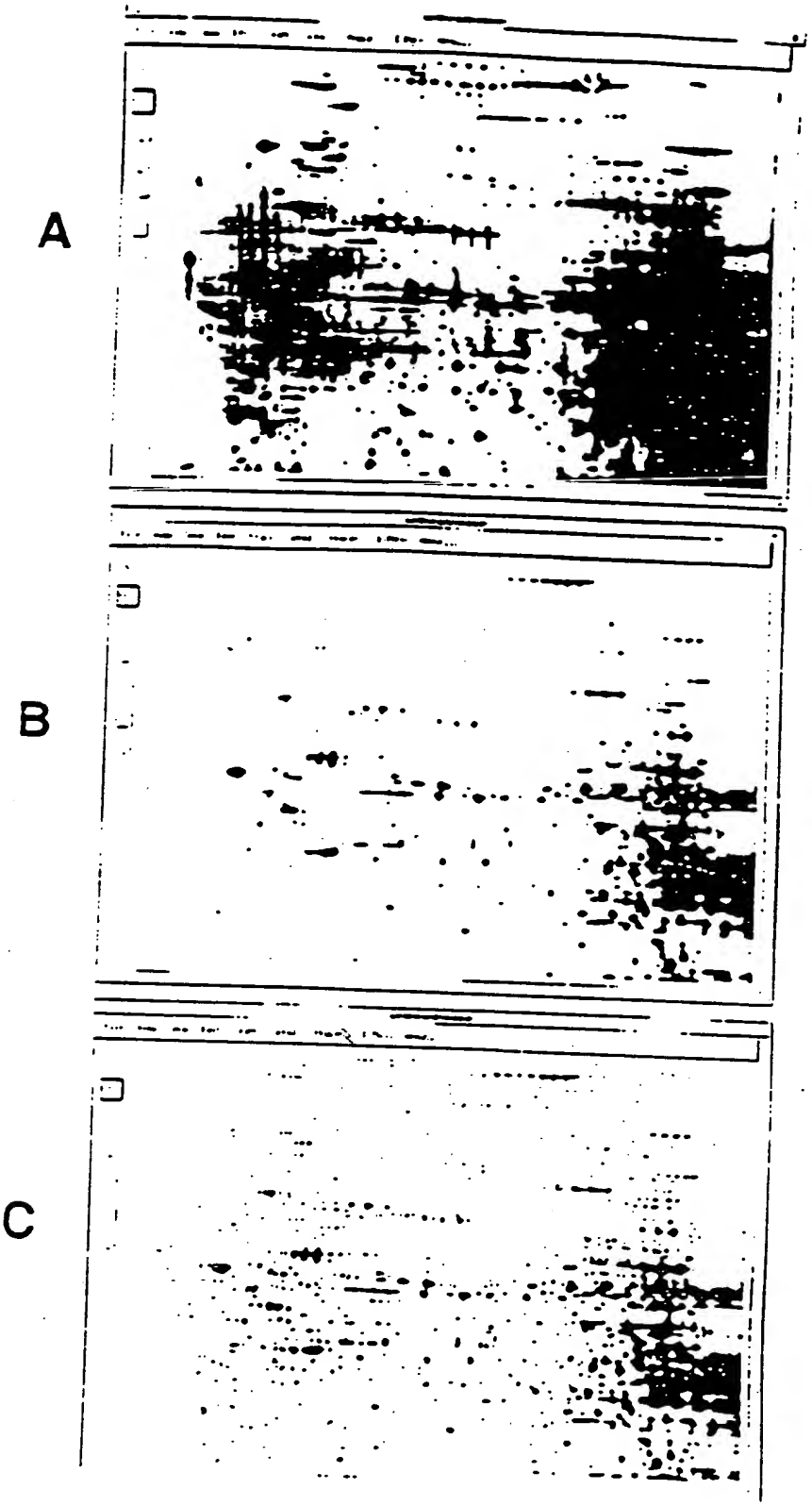

Figure 3. C mputer processing of gel images. Shown is a wide pI range 2-D separation of human liver proteins, processed by Melanie software (Appel *et al.*, 1991). (A) Original gel image as captured by laser densitometer. (B) Gel image after processing to remove streaking and background. (C) Outline definition of all spots on the gel.

CALCULATION OF PROTEIN ISOELECTRIC POINT AND MOLECULAR WEIGHT

Estimation of the isoelectric point (pI) and molecular weight (MW) of proteins from 2-D gels provides fundamental parameters for each protein, which are also of use during identification procedures (see following section). The pI and MW of proteins are recorded in 2-D gel databases. Accurate estimations of protein pI and MW can be obtained by using 20 or more known proteins on a reference map to construct standard curves of pI and molecular weight, which are then used to calculate estimated pI and MW of unknown proteins (Neidhardt *et al.*, 1989; Garrels and Franza, 1989; Van-Bogelen, Hutton and Neidhardt, 1990; Anderson and Anderson, 1991; Anderson *et al.*, 1991; Latham *et al.*, 1992). Alternatively, the MW of individual proteins blotted to PVDF can be determined very accurately by direct mass spectrometry (Eckerskorn *et al.*, 1992). Where immobilised pH gradients are used, the focusing position of proteins allows their pI to be measured within 0.15 units of that calculated from the amino acid sequence (Bjellqvist *et al.*, 1993c). It must be noted, however, that proteins carrying post-translational modifications may migrate to unexpected pI or MW positions during electrophoresis (Packer *et al.*, 1995).

SPOT QUANTITATION AND EXPRESSION ANALYSIS

A major challenge faced in proteome projects is the quantitative analysis of proteins separated by 2-D electrophoresis. The most accurate means of protein quantitation is to determine chemically the amount of each protein present by amino acid compositional analysis. However, the current method of choice for quantitative analysis of many proteins is to radiolabel samples with [^{35}S] methionine or ^{14}C amino acids, perform the 2-D electrophoresis, and measure protein levels in disintegrations per minute (dpm) or units of optical density. Quantitation is achieved either by liquid scintillation counting, or by gel image analysis where spot densities are quantitated by reference to gel calibration strips containing known amounts of radiolabelled protein or against the integrated optical density of all spots visualised (Vandekerkhove *et al.*, 1990; Celis *et al.*, 1990b; Celis and Olsen, 1994; Garrels, 1989; Latham, Garrels and Solter, 1993; Fey *et al.*, 1994). All approaches effectively allow spots to be normalised against the total disintegrations per minute loaded onto the gel. Limitations that remain with radiolabelling methods are that absolute quantitation is not achieved because all proteins have varying amounts of any amino acid, and that only easily labelled samples can be investigated. Quantitative silver staining presents an alternative (Giometti *et al.*, 1991; Harrington *et al.*, 1992; Rodriguez *et al.*, 1993; Myrick *et al.*, 1993), which when undertaken with [^{35}S]thiourea (Wallace and Saluz, 1992 a,b) is of extremely high sensitivity.

When protein spots from samples prepared under different conditions are quantitated and matched from gel to gel, it becomes possible to examine changes and patterns in protein expression. Large scale investigation of up- and down-regulation of proteins, their appearance and disappearance, can be undertaken. For example, simian virus 40 transformed human keratinocytes were shown to have 177 up-regulated and 58 down-regulated proteins compared to normal keratinocytes (Celis and Olsen, 1994); detailed synthesis profiles of 1200 proteins have been established in 1 to 4 cell mouse embryos (Latham *et al.*, 1991, 1992); and 4 proteins out of 1971 were found to be markers for

cadmium toxicity in urinary proteins (Myrick *et al.*, 1993). Complex global changes in protein expression as a result of gene disruptions have also been investigated (S. Fey and P. Mose-Larsen, Personal communication). Impressively, large gel sets showing protein expression under different conditions can be globally investigated using statistical methods that find groups of related objects within a set. For example, the REF52 rat cell line database, consisting of 79 gels from 12 experimental groups where each gel contains quantitative data for 1600 cross-matched proteins, has been analysed by cluster analysis (Garrels *et al.*, 1990). This revealed clusters of proteins that, for example, were induced or repressed similarly under simian virus 40 or adenovirus transformation, suggesting a common mechanism. Protein groups that were induced or repressed during culture growth to confluence were also found. It is obvious that the potential for investigation of cellular control mechanisms by these approaches is immense. It is equally clear that investigations of gene expression of this scale are currently technically impossible using nucleic-acid based techniques.

Table 3: Some proteome databases and their special features

Proteome database	Special features	References
E. coli gene-protein database	Gel spots linked with GenBank and Kohara clones; quantitative spot measurements under different growth conditions	VanBogelen and Neidhardt, 1991; VanBogelen *et al.*, 1992
Human heart databases	Identification of disease markers; two separate databases have been established	Baker *et al.*, 1992 Corbett *et al.*, 1993b Jungblut *et al.*, 1993
Human keratinocyte database	Extensive identifications; quantitative spot measurements of transformed cells; identification of disease markers	Celis *et al.*, 1990a Celis *et al.*, 1993 Celis and Olsen 1993
Mouse embryo database	Quantitative spot measurements through 1 to 4 cell stage	Latham *et al.*, 1991 Latham *et al.*, 1992
Mouse liver database (Argonne Protein Mapping Group)	Documents changes due to exposure to ionizing radiation and toxic chemicals	Giometti, Taylor and Tollaksen, 1992
Rat liver epithelial database	Detailed subcellular fractionation studies	Wirth *et al.*, 1991; Wirth *et al.*, 1993
Rat liver database	Extensive studies on regulation of proteins by drugs and toxic agents	Anderson and Anderson, 1991; Anderson *et al.*, 1992; Richardson, Horn and Anderson, 1993
REF 52 rat cell line database	Accessible via World Wide Web; quantitative spot measurements under different conditions	Garrels and Franza 1989 Boutell *et al.*, 1993
SWISS-2DPAGE containing human reference maps	Accessible via World Wide Web; completely integrated with SWISS-PROT and SWISS-3DIMAGE	Appel *et al.*, 1993 Hochstrasser *et al.*, 1992 Hughes *et al.*, 1993 Golaz *et al.*, 1993
Yeast Protein Database (YPD) and Yeast Electrophoretic Protein Database (YEPD)	Completely crossreferenced organism database. YPD has extensive information on over 3500 proteins; YEPD has many identifications	Garrels *et al.*, 1993

FEATURES OF PROTEOME DATABASES

Proteome projects rely heavily on computer databases to store information about all proteins expressed by an organism. 'Proteome databases' should contain detailed information of proteins already characterised elsewhere, as well as protein data from 2-D gels such as apparent pI and MW, expression level under different conditions, subcellular localisation, and information on post-translational modifications. Images of reference 2-D gels, showing protein SSP numbers and protein identifications, should also be included. Ideally, proteome databases should be accessible with Macintosh or IBM personal computers and easy to use. Some proteome databases and the areas they cover are listed in *Table 3*. Databases range from collections of annotated gels to large databases of images integrated with protein and nucleic acid sequence banks.

One example of an integrated proteome database is the suite of SWISS-PROT, SWISS-2DPAGE and SWISS-3DIMAGE databases (Appel *et al.*, 1993; Appel *et al.*, 1994; Appel, Bairoch and Hochstrasser, 1994; Bairoch and Boeckmann, 1994). The features of these three databases are listed in *Table 4*. SWISS-PROT, SWISS-2DPAGE and SWISS-3DIMAGE are accessible through the World Wide Web

Table 4: The SWISS-PROT, SWISS-2DPAGE and SWISS-3DIMAGE suite of crosslinked databases. All three databases are accessible through the World Wide Web, at URL address: http://expasy.hcuge.ch/

	SWISS-PROT	SWISS-2DPAGE	SWISS-3DIMAGE
Information	Text entries of sequence data; Citation information; taxonomic data. 38, 303 entries in Release 29	2-D gel images of: human liver, plasma, HepG2, HepG2 secreted proteins, red blood cell, lymphoma, cerebrospinal fluid, macrophage like cell line, erythroleukemia cell, platelet	Collection of 330 3-D images of proteins
Annotations	Protein function. Post translational modifications. Domains. Secondary structure. Quaternary structure. Diseases associated with protein. Sequence conflicts	Gel images where protein is found. How protein identified. Protein pI and MW. protein number; normal and pathological variants	All annotation is available in SWISS-PROT
Cross-Referenced Databases	SWISS-2DPAGE SWISS-3DIMAGE EMBL, PIR, PDB, OMIM, PROSITE, Medline, Flybase, GCRDb, MaizeDB, WormPep, DictyDB	SWISS-PROT and all other databases accessible through SWISS-PROT	SWISS-PROT and all other databases accessible through SWISS-PROT
Other Features	Navigation to other SWISS databases achieved by selecting entries with computer mouse	Gel images show position of identified proteins, or region of gel where protein should appear	Mono and stereo images available. Images can be transferred to local computer image viewing programs

(Berners-Lee *et al.*, 1992), allowing any computer connected to the internet to access the stored information and images. Navigation within and between the three databases is seamless, as all potential crosslinks are highlighted as hypertext on the display and can be selected with a computer mouse. From these databases, detailed information about a protein, including amino acid sequence and known post-translational modifications, can be obtained, the precise protein spot it corresponds to on a reference gel image can be viewed if known, and the 3-D structure of the molecule can be seen if available. References to nucleic acid and other databases are also given to provide access to information stored elsewhere.

Organism databases, containing detailed protein and nucleic acid information about a species, are becoming common as genome and proteome projects progress. These differ from nucleic acid or protein sequence databases like GenBank or SWISS-PROT because they are image based, and contain information about chromosomal map positions, transcription of genes, and protein expression patterns. The *Escherichia coli* gene-protein database (VanBogelen, Hutton and Neidhardt, 1990; VanBogelen and Neidhardt, 1991; VanBogelen *et al.*, 1992), known as the ECO2DBASE, is one example. It contains gene and protein names, 2-D gel spot information (including pI and MW estimates, and spot identification), genetic information (GenBank or EMBL codes, chromosomal location, location on Kohara clones (Kohara, Akiyama, and Isono, 1987), transcription direction of genes), and protein regulatory information (level of protein expression under different growth regimes, member of regulon or stimulon). All entries in the ECO2DBASE are also cross-referenced to the SWISS-PROT database (Bairoch and Boeckmann, 1994). It is anticipated that organism databases will soon become a standard means of storing all available information about a particular species. However there is currently no consistent manner in which organism databases are assembled, which may hamper comparisons in the future.

Identification and characterisation of proteins from 2-D gels

The number of proteins identified on a 2-D reference map determines its usefulness as a research and reference tool. As most reference maps have only a small proportion of proteins identified, a major aim of current proteome projects is to screen many proteins from 2-D maps, in order to define them as 'known' in current nucleic acid and protein databases, or as 'unknown'. Protein identification assists in confirmation of DNA open reading frames, and provides focus for DNA sequencing projects and protein characterisation efforts by pointing to proteins that are novel. Since there may be 3000–4000 proteins from a single 2-D map that require identification, the challenge in protein screening is to identify proteins quickly, with a minimum of cost and effort.

Traditionally, proteins from 2-D gels have been identified by techniques such as immunoblotting, N-terminal microsequencing, internal peptide sequencing, comigration of unknown proteins with known proteins, or by overexpression of homologous genes of interest in the organism under study (Matsudaira, 1987; Rosenfeld *et al.*, 1992; VanBogelen *et al.*, 1992; Celis *et al.*, 1993; Honore *et al.*, 1993; Garrels *et al.*, 1994). Whilst these techniques are powerful identification tools, they are too expensive or time and labour intensive to use in mass screening programs. A hierarchical approach to mass protein identification has been recently suggested as an

Table 5: Hierarchical analysis for mass screening of 2-D separated proteins blotted to membranes. Rapid and inexpensive techniques are used as a first step in protein identification, and slower, more expensive techniques are then used if necessary. Table modified from Wasinger *et al.*, 1995.

Order	Identification technique	References
1	Amino acid analysis	Jungblut *et al.*, 1992; Shaw, 1993; Hohenhm, Houthaeve and Sander, 1993; Jungblut *et al.*, 1995; Wilkins *et al.*, 1995
2	Amino acid analysis with N-terminal sequence tag	Wilkins *et al.*, submitted
3	Peptide-mass fingerprinting	Henzel *et al.*, 1993; Pappin, Hojrup and Bleasby, 1993; James *et al.*, 1993; Mann, Hojrup and Roepstorff, 1993; Yates *et al.*, 1995; Mann *et al.*, 1993; Sutton *et al.*, 1995
4	Combination of amino acid analysis and peptide mass fingerprinting	Cordwell *et al.*, 1995; Wasinger *et al.*, 1995;
5	Mass spectrometry sequence tag	Mann and Wilm, 1994
6	Extensive N-terminal Edman microsequencing	Matsudaira, 1987
7	Internal peptide Edman microsequencing	Rosenfeld *et al.*, 1992; Hellman *et al.*, 1995
8	Microsequencing by mass spectrometry (electrospray ionisation, post-source decay MALDI-TOF)	Johnson and Walsh, 1992
9	Ladder sequencing	Bartlet-Jones *et al.*, 1993

alternative to traditional approaches (*Table 5*; Wasinger *et al.*, 1995). This involves the use of rapid and cheap identification tools such as amino acid analysis and peptide mass fingerprinting as first steps in protein identification, followed by the use of slower, more expensive and time consuming identification procedures if necessary. In the construction of this hierarchy the analysis time, cost per sample and the complexity of the data created has been considered, as whilst some techniques require little machine time per sample, the analysis of data can be quite involved and time consuming. Amino acid analysis and peptide mass-fingerprinting based identification techniques in the hierarchy are discussed in detail below. For review of other protein identification techniques in *Table 5* see Patterson (1994) and Mann (1995).

PROTEIN IDENTIFICATION BY AMINO ACID COMPOSITION

There has been a revival of interest in the use of amino acid composition for identification of proteins from 2-D gels after early work by Eckerskorn *et al.* (1988). This technique uses a protein's idiosyncratic amino acid composition profile in order to identify it by comparison with theoretical compositions of proteins in databases. The amino acid composition of proteins can be determined by differential metabolic radiolabelling and quantitative autoradiography after 2-D electrophoresis (Garrels *et al.*, 1994; Frey *et al.*, 1994), or by acid hydrolysis of membrane-blotted proteins and chromatographic analysis of the resulting amino acid mixture (Eckerskorn *et al.*, 1988; Tous *et al.*, 1989; Gharahdaghi *et al.*, 1992; Jungblut *et al.*, 1992; Wilkins *et al.*, 1995). As differential metabolic labelling experiments require X-ray film or phosphor-image plate exposures of up to 140 days, and can only be undertaken with easily radiolabelled samples, the technique is not as rapid or widely applicable as chromato-

```
Spot: ECOLI-B1M
==============

Composition:

Asx: 13.2   Glx: 10.4   Ser:  5.7   His:  0.7
Gly:  8.4   Thr:  3.8   Ala:  6.7   Pro:  7.9
Tyr:  1.3   Arg:  5.0   Val:  8.0   Met:  0.3
Ile:  5.9   Leu:  8.0   Phe: 13.3   Lys:  4.4

pI estimate:    6.89  Range searched: ( 6.64,  7.14)
Mw estimate:   16800  Range searched: (13440, 20160)

Closest SWISS-PROT entries for the species ECOLI matched by AA composition:

Rank Score   Protein      pI       Mw    Description
=================================================
   1   24  PYRI_ECOLI   6.84    16989  ASPARTATE CARBAMOYLTRANSFERASE
   2   39  COAA_ECOLI   6.32    36359  PANTOTHENATE KINASE (EC 2.7.1.33)
   3   40  META_ECOLI   5.06    35723  HOMOSERINE O-SUCCINYLTRANSFERASE
   4   42  CADC_ECOLI   5.52    57812  TRANSCRIPTIONAL ACTIVATOR CADC.
   5   43  HLYC_ECOLI   8.58    19769  HEMOLYSIN C. PLASMID.

Closest SWISS-PROT entries for ECOLI with pI and Mw values in specified
range:

Rank Score   Protein      pI       Mw    Description
=================================================
   1   24  PYRI_ECOLI   6.84    16989  ASPARTATE CARBAMOYLTRANSFERASE
   2  102  TRAJ_ECOLI   6.73    17921  TRAJ PROTEIN.
   3  112  YAJG_ECOLI   6.79    19028  HYPOTHETICAL LIPOPROTEIN YAJG.
   4  140  YFJB_ECOLI   6.83    14945  HYPOTHETICAL 14.9 KD PROTEIN IN GRPE
   5  142  YAHA ECOLI   7.06    14726  HYPOTHETICAL PROTEIN IN BETT 3'REGION
```

Figure 4. Computer printout from ExPASy server where the empirical amino acid composition, estimated pI and MW of a protein from a 2-D reference map of *E. coli* were matched against all entries in SWISS-PROT for *E. coli*. The correct identification, aspartate carbamoyltransferase, is shown in bold. Low scores indicate a good match. Note how matching within a defined pI and MW range (lower set of proteins) has greatly increased the score difference between the first and second ranking proteins. This score difference gives high confidence in the identification, and is only observed where the top ranking protein is the correct identification (Wilkins *et al.*, 1995).

graphy-based analysis. Proteins blotted to PVDF membranes can be hydrolysed in 1 h at 155°C, amino acids extracted in a single brief step, and each sample automatically derivatised and separated by chromatography in under 40 minutes (Wilkins *et al.*, 1995; Ou *et al.*, 1995). In this manner, one operator can routinely analyse 100 proteins per week on one HPLC unit. This technology lends itself to automation, and it is anticipated that instruments with even greater sample throughput will be developed. When proteins have been prepared by micropreparative 2-D electrophoresis (Hanash *et al.*, 1991; Bjellqvist *et al.*, 1993b), blotted to a PVDF membrane and stained with amido black, any visible protein spot is of sufficient quantity for amino acid analysis (Cordwell *et al.*, 1995; Wasinger *et al.*, 1995; Wilkins *et al.*, 1995).

After the amino acid composition of a protein has been determined, computer programs are used to match it against the calculated compositions of proteins in databases (Eckerskorn *et al.*, 1988; Sibbald, Sommerfeldt and Argos, 1991; Jungblut *et al.*, 1992; Shaw, 1993; Hobohm, Houthaeve and Sander, 1994; Wilkins *et al.*, 1995). Matching is usually done with only 15 or 16 amino acids, as cysteine and

```
Spot ECOLI-ACS
**************

Composition:

Asx:  5.6   Glx: 10.8   Ser:  4.1   His:  2.7
Gly: 12.2   Thr:  3.8   Ala: 11.9   Pro:  3.2
Tyr:  6.0   Arg:  3.7   Val:  9.5   Met:  0.6
Ile:  5.6   Leu:  8.2   Phe:  3.2   Lys:  4.9

pI estimate:   5.99  Range searched: ( 5.74,  6.24)
Mw estimate:  45000  Range searched: (36000, 54000)

Closest SWISS-PROT entries for ECOLI with pI and Mw values in specified
range:

Rank Score   Protein    pI      Mw     N-terminal Seq.
*************************************************************
   1   21  GLYA_ECOLI  6.03   45316    M L K R E
   2   32  YUGB_ECOLI  5.86   36502    M S M I R
   3   38  GABT_ECOLI  5.78   45774    M S N S R
   4   44  YIHS_ECOLI  5.86   48018    M R I K Y
   5   45  DHE4_ECOLI  5.98   48581    M D Q T Y
   6   46  ARGD_ECOLI  5.79   43765    M A I E Q
   7   46  MURB_ECOLI  5.78   37851    M N H S L
   8   47  GLMS_ECOLI  5.98   49162    M L N R A
   9   47  ACKA_ECOLI  5.85   43290    M S S K L
  10   50  YCCX_ECOLI  6.01   37064    M E S R I
```

Figure 5. A PVDF protein spot from an *E. coli* 2-D reference map was sequenced for 4 cycles, and the same sample then subject to amino acid analysis. The N-terminal sequence was M L K R. When the amino acid composition of the spot, as well as estimated pI and MW, were matched against all entries in SWISS-PROT for *E. coli*, the above list of best matches was produced. N-terminal sequences are from SWISS-PROT for those entries. The top ranking identification of serine hydroxymethyltransferase (bold) did not show a large score difference between the first and second ranking proteins, giving little confidence in this being the correct protein identification. However, the sequence tag (M L K R) confirmed the identity of the protein as serine hydroxymethyltransferase.

tryptophan are destroyed during hydrolysis, asparagine and glutamine are deamidated to their corresponding acids, and proline is not quantitated in some analysis systems. The computer programs produce a list of best matching proteins, which are ranked by a score that indicates the match quality. Some programs allow matching to be restricted to specific 'windows' of MW and pI (Hobohm, Houthaeve and Sander, 1994; Wilkins *et al.*, 1995), and to protein database entries for one species (Jungblut *et al.*, 1992; Wilkins *et al.*, 1995). The use of such restrictions increases the power of matching. An example of protein identification by amino acid composition is shown in *Figure 4*. To date, amino acid composition has been used to identify proteins from reference maps of *Spiroplasma melliferum*, *Mycoplasma genitalium*, *E. coli*, *Saccharomyces cerevisiae*, *Dictyostelium discoideum*, human sera, human heart, human lymphocyte, and mouse brain (Cordwell *et al.*, 1995; Wasinger *et al.*, 1995; Wilkins *et al.*, 1995; Jungblut *et al.*, 1992, 1994; Garrels *et al.*, 1994; Frey *et al.*, 1994).

PROTEIN IDENTIFICATION BY AMINO ACID COMPOSITION AND N-TERMINAL SEQUENCE TAG

When samples from 2-D gels are not unambiguously identified by amino acid

composition, pI and MW, often the correct identification of that protein is amongst the top rankings of the list (Hobohm, Houthaeve and Sander, 1994; Cordwell *et al.*, 1995; Wilkins *et al.*, 1995). Taking advantage of this observation, we have used the mass spectrometry 'sequence tag' concept (Mann and Wilm, 1994) in developing a combined Edman degradation and amino acid analysis approach to protein identification (Wilkins *et al.*, submitted). This involves the N-terminal sequencing of PVDF-blotted proteins by Edman degradation for 3 or 4 cycles to create a 'sequence tag', following which the same sample is used for amino acid analysis. As only a few amino acids are removed from the protein, its composition is not significantly altered. Furthermore, since only a small amount of protein sequence is required, fast but low repetitive yield Edman degradation cycles can be used. Modifications to current procedures should allow 3 cycles to be completed in 1 h, thereby allowing the screening of 100 or more proteins per week on one automated, multi-cartridge sequenator. Amino acid composition, pI and MW of proteins are matched against databases as described above, and N-terminal sequences of best matching proteins are checked with the 'sequence tag' to confirm the protein identity (*Figure 5*). This technique will be less useful when proteins are N-terminally blocked, but as only a few N-terminal amino acids are susceptible to the acetyl, formyl, or pyroglutamyl modifications that cause blockage, this may itself provide useful information for sequence tag identification. A strength of N-terminal sequence tag and amino acid composition protein identification is that data generated are quickly and easily interpreted.

PROTEIN IDENTIFICATION BY PEPTIDE MASS FINGERPRINTING

Techniques for the identification of proteins by peptide mass fingerprinting have recently been described (Henzel *et al.*, 1993; Pappin, Højrup and Bleasby, 1993; James *et al.*, 1993; Mann, Højrup and Roepstorff, 1993; Yates *et al.*, 1993; Mortz *et al.*, 1994; Sutton *et al.*, 1995). This involves the generation of peptides from proteins using residue-specific enzymes, the determination of peptide masses, and the matching of these masses against theoretical peptide libraries generated from protein sequence databases. As proteins have different amino acid sequences, their peptides should produce characteristic 'fingerprints'.

The first step of peptide mass fingerprinting is protein digestion. Proteins within the gel matrix or bound to PVDF can be enzymatically digested *in situ*, although *in situ* gel digests are reported to produce more enzyme autodigestion products, which complicate subsequent peptide mass analysis (James *et al.*, 1993; Rasmussen *et al.*, 1994; Mortz *et al.*, 1994). The enzyme of choice for digestion is currently trypsin (of modified sequencing grade), but other enzymes (Lys-C or *S. aureus* V8 protease) have also been used (Pappin, Højrup and Bleasby, 1993). To maximise the number of peptides obtained, it is desirable for protein samples to be reduced and alkylated prior to digestion (Mortz *et al.*, 1994; Henzel *et al.*, 1993). This ensures that all disulfide bonds of the protein are broken, and produces protein conformations that are more amenable to digestion. Surprisingly, chemical digestion methods such as cyanogen bromide (methionine specific), formic acid (aspartic acid specific), and 2-(2'-nitrophenylsulfenyl)-3-methyl-3'-bromoindolenine (tryptophan specific) have not been explored as means of peptide production for mass fingerprinting, even though they are rapid and may circumvent some problems associated with enzyme digestions

(Nikodem and Fresco, 1979; Crimmins *et al.*, 1990; Vanfleteren *et al.*, 1992).

After proteins are digested, peptide masses are determined by mass spectrometry. Direct analysis of peptide mixtures can be achieved by electrospray ionisation mass spectrometry, plasma desorption mass spectrometry, or matrix assisted laser desorption ionization (MALDI) mass spectrometry techniques. MALDI is preferable because of its higher sensitivity and greater tolerance to contaminating substances from 2-D gels (James *et al.*, 1993; Mortz *et al.*, 1994; Pappin, Hojrup and Bleasby, 1993). Furthermore, recent modifications to sample preparation methods have largely solved early difficulties experienced with the calibration of MALDI spectra (Mortz *et al.*, 1994; Vorm and Mann, 1994; Vorm, Roepstorff and Mann, 1994). The high sensitivity of mass spectrometry allows a small fraction of a digest of a 1µg protein spot to be used for analysis, and analysis itself is complete in a few minutes.

A major challenge associated with peptide mass fingerprinting is data interpretation prior to computer matching against libraries of theoretical peptide digests. Spectra must be examined carefully to determine which peaks represent peptide masses of interest, as there are often enzyme autodigestion products and contaminating substances present (Henzel *et al.*, 1993; Mortz *et al.*, 1994; Rasmussen *et al.*, 1994). Furthermore, if protein alkylation and reduction has not been undertaken prior to protein digestion, peptide sequence coverage may be poor (40% to 70%), with some masses present representing disulfide bonded peptides originally present in the protein (Mortz *et al.*, 1994). For eukaryotes, a serious issue is the alteration of peptide masses by the presence of post-translational modifications (*Table* 6). The mass of the unmodified peptide alone can be very difficult to determine. Two artifactual modifications introduced by electrophoresis, an acrylamide adduct to cysteine and the oxidation of methionine, are also known to alter peptide masses (le Maire *et al.*, 1993; Hess *et al.*, 1993).

Table 6: Masses of some common post-translational modifications. Peptides carrying post-translational modifications complicate data analysis for peptide mass fingerprinting protein identification. This is especially so for protein glycosylation, which involves many different combinations of the hexosamines, hexoses, deoxyhexoses, and sialic acid

Post-translational modification	Mass change
Acetylation	+ 42.04
*Acrylamide adduct to cysteine	+71.08
Carboxylation of Asp or Glu	+44.01
Deamidation of Asn or Gln	+ 0.98
Disulfide bond formation	− 2.02
Deoxyhexoses (Fuc)	146.14
Formylation	+ 28.01
Hexosamines (GlcN, GalN)	+ 161.16
Hexoses (Glc, Gal, Man)	+ 162.14
Hydroxylation	+ 16.00
N-acetylhexosamines (GlcNAc, GalNAc)	+ 203.19
*Oxidation of Met	+ 16.00
Phosphorylation	+ 79.98
Pyroglutamic acid formed from Gln	−17.03
Sialic acid (NeuNAc)	+ 291.26
Sulfation	+ 80.06

Table modified from Finnigan LASERMAT application data sheet 5.
Asterisk * shows modifications that can arise artifactually from the 2-D electrophoresis process

A number of computer programs are available for matching peptide masses against databases (reviewed in Cottrell. 1994). Matching is usually undertaken in an interactive manner. whereby peaks of mass 500–3000 Da are selected and matched under various search parameters including MW of protein. mass accuracy of peptides. and number of missed enzyme cleavages allowed (Henzel *et al.*. 1993: Mortz *et al.*. 1994: Rasmussen *et al.*. 1994). The correct protein identity is the protein which has the most peptide masses in common with the unknown sample. Identities have been established with as few as three peptides. but unambiguous identification is thought to require a mass spectrometric map covering most peptides of the protein (Mortz *et al.*. 1994: Yates *et al.*. 1993). To date. peptide mass fingerprinting of proteins has been undertaken from the human myocardial protein and keratinocyte maps. from an *E. coli* 2-D gel. and from reference maps of *Spiroplasma melliferum* and *Mycoplasma genitalium* (Sutton *et al.*. 1995: Rasmussen *et al.*. 1994: Henzel *et al.*. 1993: Cordwell *et al.*. 1995. Wasinger *et al.*. 1995). although the technique is most powerful when used in combination with another protein identification technique (Rasmussen *et al.*. 1994: Cordwell *et al.*. 1995).

MASS SPECTROMETRY SEQUENCE TAGGING

An extension of peptide mass fingerprinting has recently been described. called peptide sequence tagging (Mann and Wilm. 1994: Mann. 1995). This uses tandem mass spectrometry (MS/MS) to initially determine the mass of peptides. then subject them to fragmentation by collision with a gas. and finally determine the mass of fragments. The resulting spectra gives information about a peptide's amino acid sequence. The fragmentation masses of peptides can rarely be used to assign a complete sequence. but it usually allows a short 'sequence tag' of 2 or 3 amino acids to be determined. This sequence tag and the original peptide mass is matched by computer against a database. providing a likely identity of the peptide and the protein it came from. The major drawback for this technique as a mass screening tool is the complexity of the mass data generated and the high level of expertise required for its interpretation. Nevertheless. it represents a useful new protein identification method which greatly increases the power of peptide mass fingerprinting protein identification.

Cross-species protein identification

Protein sequence databases continue to grow at a rapid rate. yet it is not widely appreciated that close to 90% of all information contained in current protein databases comes from only 10 species (A. Bairoch. Pers. Comm.). Fortunately. this information can be used to study proteomes of organisms that are poorly defined at the molecular level. via 2-D electrophoresis and 'cross-species' protein identification (Cordwell *et al.*. 1995: Wasinger *et al.*. 1995). This approach allows proteins from reference maps of many different species to be identified without the need for the corresponding genes to be cloned and sequenced. This is particularly true for 'housekeeping' proteins. such as enzymes involved in glycolysis. DNA manipulation and protein manufacture. which are highly conserved across species boundaries. Proteins that cannot be identified across species boundaries can then become the focus of further protein characterisation and DNA sequencing efforts.

A)

```
Protein APAI_HUMAN
******************

Asx:  8.6   Glx: 19.3   Ser:  6.3   His:  1.3
Gly:  6.2   Thr:  6.3   Ala:  8.0   Pro:  4.2
Tyr:  2.9   Arg:  6.7   Val:  5.5   Met:  1.3
Ile:  0.0   Leu: 15.5   Phe:  2.5   Lys:  8.8

pI Range: no range specified
Mw Range: no range specified

The closest SWISS-PROT entries are:

Rank Score   Protein    (pI       Mw)  Description
*************************************************
   1     0 APAI_HUMAN   5.27     28078  APOLIPOPROTEIN A-I.
   2     4 APAI_MACFA   5.43     28005  APOLIPOPROTEIN A-I.
   3    12 APAI_RABIT   5.15     27836  APOLIPOPROTEIN A-I.
   4    14 APAI_BOVIN   5.36     27549  APOLIPOPROTEIN A-I.
   5    14 APAI_CANFA   5.10     27467  APOLIPOPROTEIN A-I.
   6    18 APAI_MOUSE   5.62     27922  APOLIPOPROTEIN A-I.
   7    26 APAI_PIG     5.19     27598  APOLIPOPROTEIN A-I.
   8    27 APAI_CHICK   5.26     27966  APOLIPOPROTEIN A-I.
   9    37 DYNA_CHICK   5.44    217762  DYNACTIN, 217 KD ISOFORM.
  10    39 APA4_HUMAN   5.18     43374  APOLIPOPROTEIN A-IV.
```

B)

```
Reagent: Trypsin  MW filter: 10%

Scan using fragment mws of:

1953  1933  1731  1613  1401  1387
1301  1283  1252  1235  1231  1215
1031   996   873   831   823   781
 732   706

No. of database entries scanned = 72018

1  . APAI_HUMAN   APOLIPOPROTEIN A-I (APO-AI). - HOMO SAPIENS
2  . APAI_MACFA   APOLIPOPROTEIN A-I (APO-AI). - MACACA FASCICULARIS
3  . APAI_PAPHA   APOLIPOPROTEIN A-I (APO-AI). - PAPIO HAMADRYAS
4  . B41845       orf B - Treponema denticola
5  . APAI_CANFA   APOLIPOPROTEIN A-I (APO-AI). - CANIS FAMILIARIS (DOG).
6  . S30947       hypothetical protein 1 - Azotobacter vinelandii
7  . HS2C_PEA     CHLOROPLAST HEAT SHOCK PROTEIN PRECURSOR. - PISUM SATIVU
8  . S20724       Tropomyosin - African clawed frog
9  . HIVV2354     HIVV2354 premature term. at 793 - Human immunodeficiency
10 . TRAJ_ECOLI   TRAJ PROTEIN. - ESCHERICHIA COLI.
```

Figure 6. Theoretical cross-species matching of human apolipoprotein A-I by amino acid composition and tryptic peptides. When an unknown protein is analysed, best ranking proteins from both techniques can be compared. If the same protein type is observed in both lists, there is high confidence in this being the identity of the unknown molecule (Cordwell *et al.*, 1995). (A) Output of ExPASy server (Appel, Bairoch and Hochstrasser, 1994) where the true amino acid composition of apolipoprotein A-I was matched against all entries in the SWISS-PROT database, without pI or MW windows. Seven of the top 10 matching proteins were apolipoprotein A-I of different species. (B) Output of MOWSE peptide mass fingerprinting program (Pappin, Hojrup and Bleasby, 1993) where true tryptic peptides of human apolipoprotein A-I were matched against the OWL database, using MW window of 10%. Four of the top ten matching proteins were apolipoprotein A-I from different species.

Rapid cross-species identification of proteins from 2-D reference maps can be undertaken with amino acid composition or peptide mass fingerprinting methods (*Figure 6*), but these techniques alone may not identify proteins unambiguously when phylogenetic cross-species distances are great or analysis data is of poor quality (Yates *et al.*, 1993; Shaw, 1993; Cordwell *et al.*, 1995). However, very high confidence in protein identities can be achieved when lists of best-matching proteins generated by both techniques are compared (Cordwell *et al.*, 1995; Wasinger *et al.*, 1995). The correct identification is found when the same protein is ranked highly in lists of best matches generated by both techniques. This method has allowed approximately 120 proteins from the reference map of the mollicute *Spiroplasma melliferum*, representing approximately one quarter of the proteome, to be confidently identified by reference to protein information from other species (S. Cordwell, Personal Communication). When cross-species protein identification is to be undertaken, it should be noted that the molecular weight of a protein type across species is usually highly conserved, but that protein pI can vary by more than 2 units (Cordwell *et al.*, 1995). Accurate molecular weight determination by direct mass spectrometry of proteins blotted to PVDF (Eckerskorn *et al.*, 1992) should therefore be a useful additional parameter for cross-species protein identification.

CHARACTERISATION OF POST-TRANSLATIONAL MODIFICATIONS

Many proteins are modified after translation. Such post-translational modifications, including glycosylation, phosphorylation, and sulfation (see *Table 6*), are usually necessary for protein function or stability. Some abnormal modifications are associated with disease (Duthel and Revol, 1993; Ghosh *et al.*, 1993; Yamashita *et al.*, 1993). In proteome studies, post-translational modifications can be examined on all proteins present, or on individual spots. Studies on all proteins provide an indication of which proteins may carry a certain type of modification. For example, 2-D gel analysis of cell cultures grown in the presence of [^{3}H] mannose or [^{32}P] phosphate gives an indication of which proteins carry glycans containing mannose, and which proteins are phosphorylated (Garrels and Franza, 1989). Lectin binding studies of 2-D gels blotted to PVDF or nitrocellulose provide information on the saccharides, if any, that are carried by proteins present (Gravel *et al.*, 1994).

When individual proteins of interest carrying post-translational modifications have been found, micropreparative 2-D electrophoresis can be used to purify them in microgram quantities (Hanash *et al.*, 1991; Bjellqvist *et al.*, 1993b). If protein isoforms of similar MW and pI are to be studied, focusing with narrow range pI gradients (1 pH unit) can provide greater separation and resolution. After electrophoresis, the type and degree of protein phosphorylation can be investigated (Murthy and Iqbal, 1991; Gold *et al.*, 1994), monosaccharide composition can be determined (Weitzhandler *et al.*, 1993; Packer *et al.*, 1995), and the structure and exact site of glycoamino acids can be investigated by either Edman degradation based techniques or by mass spectrometry (Pisano *et al.*, 1993; Huberty *et al.*, 1993; Carr, Huddleston and Bean, 1993). With further development of rapid techniques, investigation of phosphorylation and monosaccharides by chromatographic or mass spectrometric means is likely to become a routine step in the characterisation of post-translational modifications of proteins from reference maps.

The status of proteome projects

Many technical aspects of proteome research have already been discussed in this review, but an overview of the status of proteome projects has not yet been presented. Advances in proteome projects will initially rely on progress in genome sequencing initiatives, to enable an identity, amino acid sequence, or function to be assigned to each protein spot. *Table 7* shows genome size, proteome size, and the number of proteins already defined for a number of model organisms. This indicates that whilst genome sequencing programs for *E. coli* and *S. cerevisiae* are advanced, the massive size of some other genomes (and especially the human genome) means that their complete nucleotide sequences are unlikely to be available for many years. Because of this, 2-D reference maps and proteome projects of single cell organisms like *Mycoplasma sp.*, *E. coli* and *S. cerevisiae* will be the most detailed (Cordwell *et al.*, 1995; Wasinger *et al.*, 1995; Vanbogelen *et al.*, 1992; Garrels *et al.*, 1994), and complete maps of other organisms will take longer to construct. However, the use of cross-species protein identification techniques will allow proteomes of many prokaryotes and simple eukaryotes to be partially defined in reference to *E. coli* and *S. cerevisiae*.

Table 7: Estimated genome size, estimated proteome size, number of protein sequences in SWISS-PROT Release 31 (March, 1995), and approximate number of proteins of known identity on 2-D reference maps for some model organisms. Genome size data from Smith (1994), and total protein data from Bird (1995). Genome sequencing projects of *E. coli* and *S. cerevisiae* will probably be complete in 1996.

Species Name	Haploid genomesSize (million bp)	Estimated proteome size (total proteins)	Protein entries in SWISS PROT	Proteins annotated on 2-D Maps
Mycoplasma species	0.6–0.8	400–600	100	> 100
Escherichia coli	4.8	4000	3170	> 300
Saccharomyces cerevisiae	13.5	6000	3160	> 100
Dictyostelium discoideum	70	12500	204	–
Arabidopsis thaliana	70	14000	270	–
Caenorhabditis elegans	80	17800	703	–
Homo sapiens	2900	60000–80000	3326	> 1000

The study of vertebrate proteomes and vertebrate development is a phenomenal undertaking in comparison to the investigation of single cell organisms. This is because vast numbers of proteins are developmentally expressed, each body tissue has hundreds of unique proteins, and there are numerous tissue types. However, it is estimated that at least 35% of proteins in vertebrate cells will be conserved from tissue to tissue, constituting the 'housekeeping' proteins (Bird, 1995), with the remainder of proteins constituting a set that are specific to a cell type. Providing that standardised electrophoretic conditions are used, reference maps from many tissues of one organism can be superimposed in gel databases (e.g. Hochstrasser *et al.*, 1992). This accelerates the definition of the 'housekeeping' proteins, as well as sets of proteins that are unique to different tissue types. Such studies may, however, be complicated by post-translational modifications, which can differ on the same gene product in different tissues. Proteins that remain unknown after identification procedures will be useful in providing focus for nucleic acid sequencing initiatives.

FUTURE DIRECTIONS OF PROTEOME PROJECTS

This review has described recent advances in the area of proteome research. It has illustrated how new developments of older techniques (2-D electrophoresis and amino acid analysis) as well as the applications of new technology (mass spectrometry) have greatly widened the choice of tools the biologist and protein chemist has for the separation, identification and analysis of complex mixtures of proteins. This has made possible the establishment of detailed reference maps for organisms, which are becoming the method of choice for the definition of tissues or whole cells, and the investigation of gene expression therein.

Proteome projects are already impacting on the dogma of molecular biology that DNA sequence constitutes the definition of an organism. For example, the proteomes of different tissues of a single organism are often significantly different. Similarly, cross-species identification of proteins (for example the identification of proteins from *Candida albicans* by comparison with *S. cerevisiae*) can open up studies on organisms that are poorly molecularly defined. As cross-species identification can proceed at a pace orders of magnitude faster than a genome project in terms of defining the gene and protein complement of organims, the need for the DNA sequencing of genomes will be avoided, and emphasis placed on those found to be novel.

Just as genome sequencing is not an end in itself, neither is an annotated 2-D protein reference map of an organism, nor indeed the identification of proteins in a proteome. So whilst an immediate aim of proteome projects is to screen proteins in reference maps, this will lead to expression studies and characterisation of post-translational modifications. The challenge that then needs to be addressed is the investigation of structure and function of proteins in a proteome. The magnitude of this is illustrated by the fact that over half the open reading frames identified in *S. cerevisiae* chromosome III were initially of no known function (Oliver *et al.*, 1992). Structural and functional studies will be an undertaking just as formidable as genome studies are now and proteome projects are becoming, but will lead to an unimaginably detailed understanding of how living organisms are constructed and how they operate.

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Differential gene expression in drug metabolism and toxicology: practicalities, problems and potential

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1. An important feature of the work of many molecular biologists is identifying which genes are switched on and off in a cell under different environmental conditions or subsequent to xenobiotic challenge. Such information has many uses, including the deciphering of molecular pathways and facilitating the development of new experimental and diagnostic procedures. However, the student of gene hunting should be forgiven for perhaps becoming confused by the mountain of information available as there appears to be almost as many methods of discovering differentially expressed genes as there are research groups using the technique.

2. The aim of this review was to clarify the main methods of differential gene expression analysis and the mechanistic principles underlying them. Also included is a discussion on some of the practical aspects of using this technique. Emphasis is placed on the so-called 'open' systems, which require no prior knowledge of the genes contained within the study model. Whilst these will eventually be replaced by 'closed' systems in the study of human, mouse and other commonly studied laboratory animals, they will remain a powerful tool for those examining less fashionable models.

3. The use of suppression-PCR subtractive hybridization is exemplified in the identification of up- and down-regulated genes in rat liver following exposure to phenobarbital, a well-known inducer of the drug metabolizing enzymes.

4. Differential gene display provides a coherent platform for building libraries and microchip arrays of 'gene fingerprints' characteristic of known enzyme inducers and xenobiotic toxicants, which may be interrogated subsequently for the identification and characterization of xenobiotics of unknown biological properties.

Introduction

It is now apparent that the development of almost all cancers and many non-neoplastic diseases are accompanied by altered gene expression in the affected cells compared to their normal state (Hunter 1991, Wynford-Thomas 1991, Vogelstein and Kinzler 1993, Semenza 1994, Cassidy 1995, Kleinjan and Van Hegningen 1998). Such changes also occur in response to external stimuli such as pathogenic micro-organisms (Rohn *et al.* 1996, Singh *et al.* 1997, Griffin and Krishna 1998, Lunney 1998) and xenobiotics (Sewall *et al.* 1995, Dogra *et al.* 1998, Ramana and Kohli 1998), as well as during the development of undifferentiated cells (Hecht 1998, Rudin and Thompson 1998, Schneider-Maunoury *et al.* 1998). The potential medical and therapeutic benefits of understanding the molecular changes which occur in any given cell in progressing from the normal to the 'altered' state are enormous. Such profiling essentially provides a 'fingerprint' of each step of a

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cell's development or response and should help in the elucidation of specific and sensitive biomarkers representing, for example, different types of cancer or previous exposure to certain classes of chemicals that are enzyme inducers.

In drug metabolism, many of the xenobiotic-metabolizing enzymes (including the well-characterized isoforms of cytochrome P450) are inducible by drugs and chemicals in man (Pelkonen *et al.* 1998), predominantly involving transcriptional activation of not only the cognate cytochrome P450 genes, but additional cellular proteins which may be crucial to the phenomenon of induction. Accordingly, the development of methodology to identify and assess the full complement of genes that are either up- or down-regulated by inducers are crucial in the development of knowledge to understand the precise molecular mechanisms of enzyme induction and how this relates to drug action. Similarly, in the field of chemical-induced toxicity, it is now becoming increasingly obvious that most adverse reactions to drugs and chemicals are the result of multiple gene regulation, some of which are causal and some of which are casually-related to the toxicological phenomenon *per se*. This observation has led to an upsurge in interest in gene-profiling technologies which differentiate between the control and toxin-treated gene pools in target tissues and is, therefore, of value in rationalizing the molecular mechanisms of xenobiotic-induced toxicity. Knowledge of toxin-dependent gene regulation in target tissues is not solely an academic pursuit as much interest has been generated in the pharmaceutical industry to harness this technology in the early identification of toxic drug candidates, thereby shortening the developmental process and contributing substantially to the safety assessment of new drugs. For example, if the gene profile in response to say a testicular toxin that has been well-characterized *in vivo* could be determined in the testis, then this profile would be representative of all new drug candidates which act via this specific molecular mechanism of toxicity, thereby providing a useful and coherent approach to the early detection of such toxicants. Whereas it would be informative to know the identity and functionality of all genes up/down regulated by such toxicants, this would appear a longer term goal, as the majority of human genes have not yet been sequenced, far less their functionality determined. However, the current use of gene profiling yields a *pattern* of gene changes for a xenobiotic of unknown toxicity which may be matched to that of well-characterized toxins, thus alerting the toxicologist to possible *in vivo* similarities between the unknown and the standard, thereby providing a platform for more extensive toxicological examination. Such approaches are beginning to gain momentum, in that several biotechnology companies are commercially producing 'gene chips' or 'gene arrays' that may be interrogated for toxicity assessment of xenobiotics. These chips consist of hundreds/thousands of genes, some of which are degenerate in the sense that not all of the genes are mechanistically-related to any one toxicological phenomenon. Whereas these chips are useful in broad-spectrum screening, they are maturing at a substantial rate, in that gene arrays are now becoming more specific, e.g. chips for the identification of changes in growth factor families that contribute to the aetiology and development of chemically-induced neoplasias.

Although documenting and explaining these genetic changes presents a formidable obstacle to understanding the different mechanisms of development and disease progression, the technology is now available to begin attempting this difficult challenge. Indeed, several 'differential expression analysis' methods hav be n developed which facilitate the identification of gen products that demonstrate

altered expression in cells of one population compared to another. These methods have been used to identify differential gene expression in many situations, including invading pathogenic microbes (Zhao *et al.* 1998), in cells responding to extracellular and intracellular microbial invasion (Duguid and Dinauer 1990, Ragno *et al.* 1997, Maldarelli *et al.* 1998), in chemically treated cells (Syed *et al.* 1997, Rockett *et al.* 1999), neoplastic cells (Liang *et al.* 1992, Chang and Terzaghi-Howe 1998), activated cells (Gurskaya *et al.* 1996, Wan *et al.* 1996), differentiated cells (Hara *et al.* 1991, Guimaraes *et al.* 1995a, b), and different cell types (Davis *et al.* 1984, Hedrick *et al.* 1984, Xhu *et al.* 1998). Although differential expression analysis technologies are applicable to a broad range of models, perhaps their most important advantage is that, in most cases, absolutely no prior knowledge of the specific genes which are up- or down-regulated is required.

The field of differential expression analysis is a large and complex one, with many techniques available to the potential user. These can be categorized into several methodological approaches, including:

(1) Differential screening,
(2) Subtractive hybridization (SH) (includes methods such as chemical cross-linking subtraction—CCLS, suppression-PCR subtractive hybridization—SSH, and representational difference analysis—RDA),
(3) Differential display (DD),
(4) Restriction endonuclease facilitated analysis (including serial analysis of gene expression—SAGE—and gene expression fingerprinting—GEF),
(5) Gene expression arrays, and
(6) Expressed sequence tag (EST) analysis.

The above approaches have been used successfully to isolate differentially expressed genes in different model systems. However, each method has its own subtle (and sometimes not so subtle) characteristics which incur various advantages and disadvantages. Accordingly, it is the purpose of this review to clarify the mechanistic principles underlying the main differential expression methods and to highlight some of the broader considerations and implications of this very powerful and increasingly popular technique. Specifically, we will concentrate on the so-called 'open' systems, namely those which do not require any knowledge of gene sequences and, therefore, are useful for isolating unknown genes. Two 'closed' systems (those utilising previously identified gene sequences), EST analysis and the use of DNA arrays, will also be considered briefly for completeness. Whilst emphasis will often be placed on suppression PCR subtractive hybridization (SSH, the approach employed in this laboratory), it is the aim of the authors to highlight, wherever possible, those areas of common interest to those who use, or intend to use, differential gene expression analysis.

Differential cDNA library screening (DS)

Despite the development of multiple technological advances which have recently brought the field of gene expression profiling to the forefront of molecular analysis, recognition of the importance of differential gene expression and characterization of differentially expressed genes has existed for many years. One of the original approaches used to identify such genes was described 20 years ago by St John and Davis (1979). These authors developed a method, termed 'differential plaque filter

hybridization', which was used to isolate galactose-inducible DNA sequences from yeast. The theory is simple: a genomic DNA library is prepared from normal, unstimulated cells of the test organism/tissue and multiple filter replicas are prepared. These replica blots are probed with radioactively (or otherwise) labelled complex cDNA probes prepared from the control and test cell mRNA populations. Those mRNAs which are differentially expressed in the treated cell population will show a positive signal only on the filter probed with cDNA from the treated cells. Furthermore, labelled cDNA from different test conditions can be used to probe multiple blots, thereby enabling the identification of mRNAs which are only up-regulated under certain conditions. For example, St John and Davis (1979) screened replica filters with acetate-, glucose- and galactose-derived probes in order to obtain genes induced specifically by galactose metabolism. Although groundbreaking in its time this method is now considered insensitive and time-consuming, as up to 2 months are required to complete the identification of genes which are differentially expressed in the test population. In addition, there is no convenient way to check that the procedure has worked until the whole process has been completed.

Subtractive Hybridization (SH)

The developing concept of differential gene expression and the success of early approaches such as that described by St John and Davis (1979) soon gave rise to a search for more convenient methods of analysis. One of the first to be developed was SH, numerous variations of which have since been reported (see below). In general, this approach involves hybridization of mRNA/cDNA from one population (tester) to excess mRNA/cDNA from another (driver), followed by separation of th unhybridized tester fraction (differentially expressed) from the hybridized common sequences. This step has been achieved physically, chemically and through the use of selective polymerase chain reaction (PCR) techniques.

Physical separation

Original subtractive hybridization technology involved the physical separation of hybridized common species from unique single stranded species. Several methods of achieving this have been described including hydroxyapatite chromatography (Sargent and Dawid 1983), avidin-biotin technology (Duguid and Dinauer 1990) and oligodT-latex separation (Hara *et al.* 1991). In the first approach, common mRNA species are removed by cDNA (from test cells)-mRNA (from control c lls) subtractive hybridization followed by hydroxyapatite chromatography, as hydroxyapatite specifically adsorbs the cDNA-mRNA hybrids. The unabsorbed cDNA is then used either for the construction of a cDNA library of differentially expr ss d genes (Sargent and Dawid 1983, Schneider *et al.* 1988) or directly as a probe to screen a preselected library (Zimmerman *et al.* 1980, Davis *et al.* 1984, Hedrick *et al.* 1984). A schematic diagram of the procedure is shown in figure 1.

Less rigorous physical separation procedures coupled with sensitivity enhancing PCR steps were later developed as a means to overcome some of the problems encounter d with the hydroxyapatite procedure. For example, Daguid and Dinauer (1990) described a method of subtraction utilizing biotin-affinity systems as a means to remove hybridized common sequences. In this process, both the control and tester mRNA populations are first converted to cDNA and an adaptor ('oligovector',

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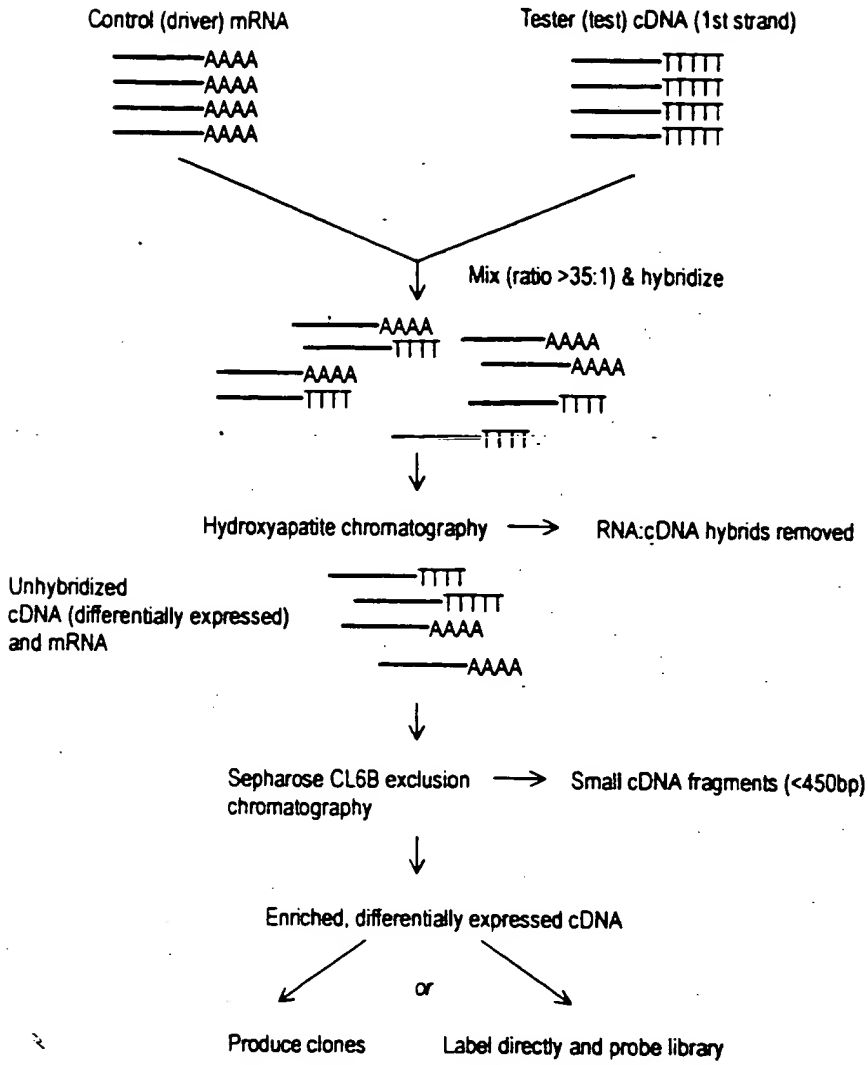

Figure 1. The hydroxyapatite method of subtractive hybridization. cDNA derived from the treated/altered (tester) population is mixed with a large excess of mRNA from the control (driver) population. Following hybridization, mRNA-cDNA hybrids are removed by hydroxyapatite chromatography. The only cDNAs which remain are those which are differentially expressed in the treated/altered population. In order to facilitate the recovery of full length clones, small cDNA fragments are removed by exclusion chromatography. The remaining cDNAs are then cloned into a vector for sequencing, or labelled and used directly to probe a library, as described by Sargent and Dawid (1983).

containing a restriction site) ligated to both sides. Both populations are then amplified by PCR, but the driver cDNA population is subsequently digest d with the adaptor-containing restriction endonuclease. This serves to cleave the oligo-vector and reduce the amplification potential of the control population. The dig st d control population is then biotinylated and an excess mixed with tester cDNA. Following denaturation and hybridization, the mix is applied to a biocytin column (streptavidin may also be used) to remove th control population, including heteroduplexes formed by annealing of common sequences from the tester population. The procedure is repeated several times following the addition of fresh

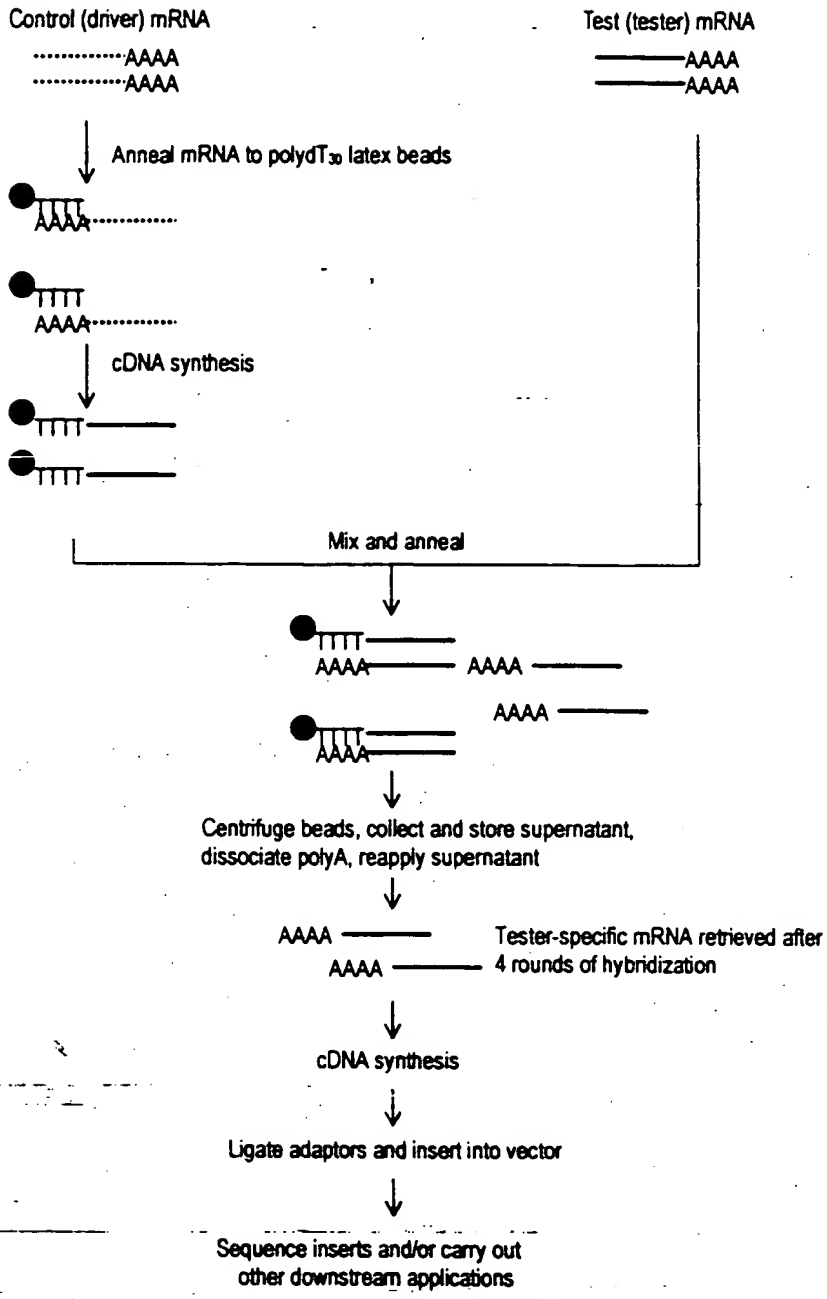

Figure 2. The use of oligodT_{30} latex to perform subtractive hybridization. mRNA extracted from the control (driver) population is converted to anchored cDNA using polydT oligonucleotides attached to latex beads. mRNA from the treated/altered (tester) population is repeatedly hybridized against an excess of the anchored driver cDNA. The final population of mRNA is tester specific and can be converted into cDNA for cloning and other downstream applications, as described by Hara *et al.* (1991).

control cDNA. In order to further enrich those species differentially expressed in the tester cDNA, the subtracted tester population is amplified by PCR following every second subtraction cycle. After six cycles of subtraction (three reamplification steps) the reaction mix is ligated into a vector for further analysis.

In a slightly different approach, Hara *et al.* (1991) utilized a method whereby oligo(dT_{30}) primers attached to a latex substrate are used to first capture mRNA extracted from the control population. Following 1st strand cDNA synthesis, the RNA strand of the heteroduplexes is removed by heat denaturation and centrifugation (the cDNA-oligotex-dT_{30} forms a pellet and the supernatant is removed). A quantity of tester mRNA is then repeatedly hybridized to the immobilized control (driver) cDNA (which is present in 20-fold excess). After several rounds of hybridization the only mRNA molecules left in the tester mRNA population are those which are not found in the driver cDNA-oligotex-dT_{30} population. These tester-specific mRNA species are then converted to cDNA and, following the addition of adaptor sequences, amplified by PCR. The PCR products are then ligated into a vector for further analysis using restriction sites incorporated into the PCR primers. A schematic illustration of this subtraction process is shown in figure 2.

However, all these methods utilising physical separation have been described as inefficient due to the requirement for large starting amounts of mRNA, significant loss of material during the separation process and a need for several rounds of hybridization. Hence, new methods of differential expression analysis have recently been designed to eliminate these problems.

Chemical Cross-Linking Subtraction (CCLS)

In this technique, originally described by Hampson *et al.* (1992), driver mRNA is mixed with tester cDNA (1st strand only) in a ratio of $> 20:1$. The common sequences form cDNA:mRNA hybrids, leaving the tester specific species as single stranded cDNA. Instead of physically separating these hybrids, they are inactivated chemically using 2,5 diaziridinyl-1,4-benzoquinone (DZQ). Labelled probes are then synthesized from the remaining single stranded cDNA species (unreacted mRNA species remaining from the driver are not converted into probe material due to specificity of Sequenase T7 DNA polymerase used to make the probe) and used to screen a cDNA library made from the tester cell population. A schematic diagram of the system is shown in figure 3.

It has been shown that the differentially expressed sequences can be enriched at least 300-fold with one round of subtraction (Hampson *et al.* 1992), and that the technique should allow isolation of cDNAs derived from transcripts that are present at less than 50 copies per cell. This equates to genes at the low end of intermediate abundance (see table 1). The main advantages of the CCLS approach are that it is rapid, technically simple and also produces fewer false positives than other differential expression analysis methods. However, like the physical separation protocols, a major drawback with CCLS is the large amount of starting material required (at least 10 μg RNA). Consequently, the technique has recently been refined so that a renewable source of RNA can be generated. The degenerate random oligonucleotide primed (DROP) adaptation (Hampson *et al.* 1996, Hampson and Hampson 1997) uses random hexanucleotide sequences to prime solid phase-synthesized cDNA. Since each primer includes a T7 polymerase promotor sequence

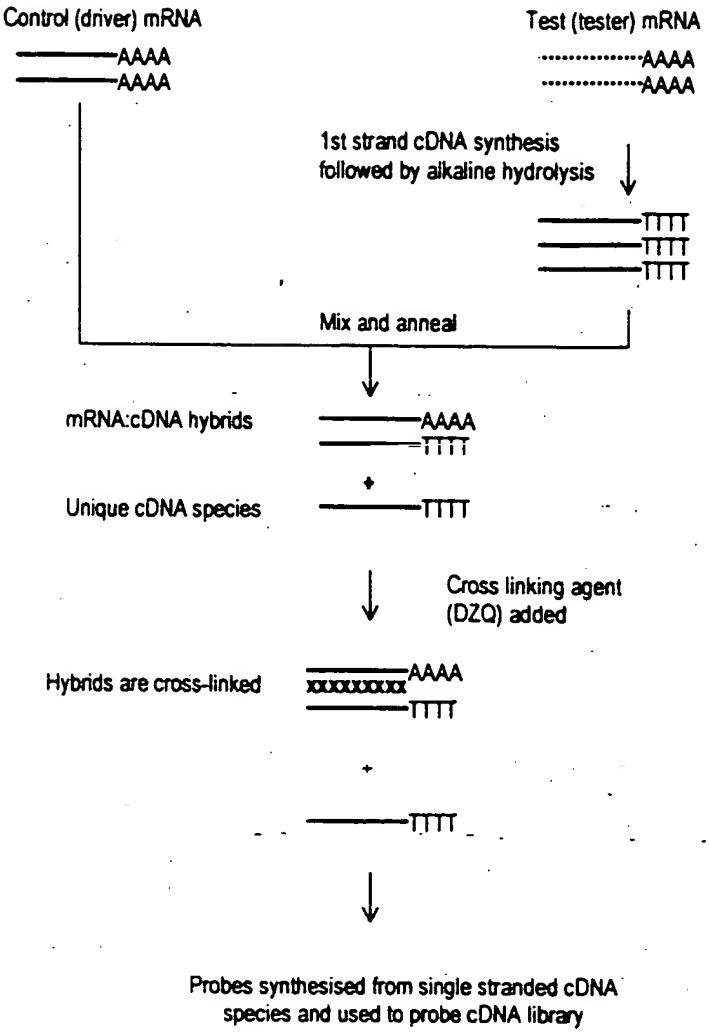

Figure 3. Chemical cross-linking subtraction. Excess driver mRNA is mixed with 1st strand tester cDNA. The common sequences form mRNA:cDNA hybrids which are cross linked with 2,5 diaziridinyl-1,4-benzoquinone (DZQ) and the remaining cDNA sequences are differentially expressed in the tester population. Probes are made from these sequences using Sequenase 2.0 DNA polymerase, which lacks reverse transcriptase activity and, therefore, does not react with the remaining mRNA molecules from the driver. The labelled probes are then used to screen a cDNA library for clones of differentially expressed sequences. Adapted from Walter *et al.* (1996), with permission.

Table 1. The abundance of mRNA species and classes in a typical mammalian cell.

mRNA class	Copies of each species/cell	No. of mRNA species in class	Mean % of each species in class	Mean mass (ng) of each species/μg total RNA
Abundant	12000	4	3.3	1.65
Intermediate	300	500	0.08	0.04
Rare	15	11000	0.004	0.002

Modified from Bertioli *et al.* (1995).

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0.04
0.002

at the 5′ end, the final pool of random cDNA fragments is a PCR-renewable cDNA population which is representative of the expressed gene pool and can be used to synthesize sense RNA for use as driver material. Furthermore, if the final pool of random cDNA fragments is reamplified using biotinylated T7 primer and random hexamer, the product can be captured with streptavidin beads and the antisense strand eluted for use as tester. Since both target and driver can be generated from the same DROP product, subtraction can be performed in both directions (i.e. for up- and down-regulated species) between two different DROP products.

Representational Difference Analysis (RDA)

RDA of cDNA (Hubank and Schatz 1994) is an extension of the technique originally applied to genomic DNA as a means of identifying differences between two complex genomes (Lisitsyn *et al.* 1993). It is a process of subtraction and amplification involving subtractive hybridization of the tester in the presence of excess driver. Sequences in the tester that have homologues in the driver are rendered unamplifiable, whereas those genes expressed only in the tester retain the ability to be amplified by PCR. The procedure is shown schematically in figure 4.

In essence, the driver and tester mRNA populations are first converted to cDNA and amplified by PCR following the ligation of an adaptor. The adaptors are then removed from both populations and a new (different) adaptor ligated to the amplified tester population only. Driver and tester populations are next melted and hybridized together in a ratio of 100:1. Following hybridization, only tester:tester homohybrids have 5′ adaptors at each end of the DNA duplex and can, thus, be filled in at both 3′ ends. Hence, only these molecules are amplified exponentially during the subsequent PCR step. Although tester:driver heterohybrids are present, they only amplify in a linear fashion, since the strand derived from the driver has no adaptor to which the primer can bind. Driver:driver heterohybrids have n adaptors and, therefore, are not amplified. Single stranded molecules are digested with mung bean nuclease before a further PCR-enrichment of the tester:tester homohybrids. The adaptors on the amplified tester population are then replaced and the whole process repeated a further two or three times using an increasing excess of driver (Hubank and Shatz used a tester:driver ratio of 1:400, 1:80000 and 1:800000 for the second, third and fourth hybridizations, respectively). Different adaptors are ligated to the tester between successive rounds of hybridization and amplification to prevent the accumulation of PCR products that might interfere with subsequent amplifications. The final display is a series of differentially expressed gene products easily observable on an ethidium bromide gel.

The main advantages of RDA are that it offers a reproducible and sensitive approach to the analysis of differentially expressed genes. Hubank and Schatz (1994) reported that they were able to isolate genes that were differentially expressed in substantially less than 1% of the cells from which the tester is derived. Perhaps the main drawback is that multiple rounds of ligation, hybridization, amplifiation and digestion are required. The procedure is, therefore, lengthier than many other differential display approaches and provides more opportunity for operator-induced error to occur. Although the generation of false positives has been noted, this has been solved to some degree by O'Neill and Sinclair (1997) through the use of HPLC-purifi d adaptors. These are free of the truncated adaptors which appear to be a major source of the false positive bands. A very similar technique to RDA, termed linker capture subtracti n (LCS) was described by Yang and Sytowski (1996).

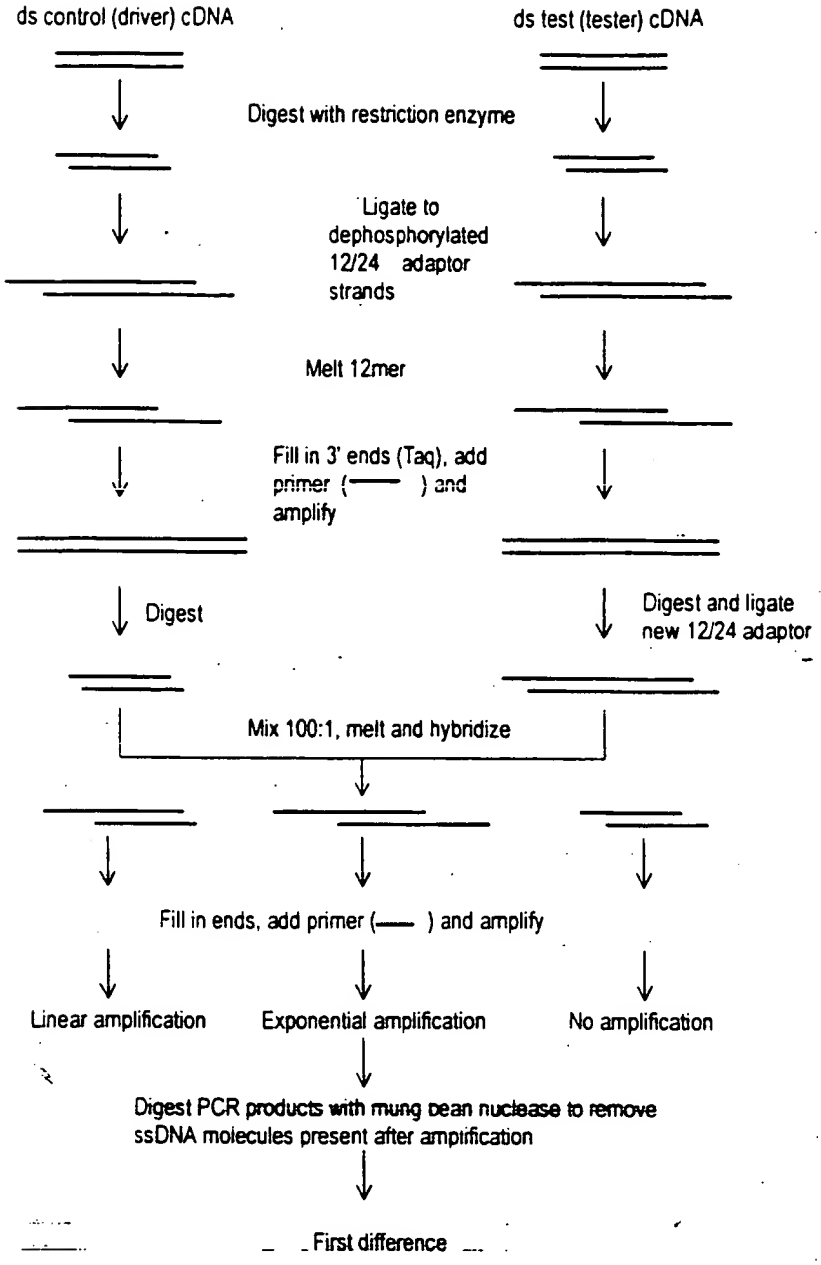

Figure 4. The representational difference analysis (RDA) technique. Driver and tester cDNA are digested with a 4-cutter restriction enzyme such as *Dpn*II. The 1st set of 12/24 adaptor strands (oligonucleotides) are ligated to each other and the digested cDNA products. The 12mer is subsequently melted away and the 3'ends filled in using Taq DNA polymerase. Each cDNA population is then amplified using PCR, following which the 1st set of adaptors is removed with *Dpn*II. A second set of 12/24 adaptor strands is then added to the amplified tester cDNA population, after which the tester is hybridized against a large excess of driver. The 12mer adaptors are melted and the 3' ends filled in as before. PCR is carried out with primers identical to the new 24mer adaptor. Thus, the only hybridization products which are exponentially amplified are those which are tester:tester combinations. Following PCR, ssDNA products are removed with mung bean nuclease, leaving the 'first difference product'. This is digested and a third set of 12/24 adaptors added before repeating the subtraction process from the hybridization stage. The process is repeated to the 3rd or 4th difference product, as described by Lisitsyn *et al.* (1993) and Hubank and Schatz (1994).

Suppression PCR Subtractive Hybridization (SSH)

The most recent adaptation of the SH approach to differential expression analysis was first described by Diatchenko *et al.* (1996) and Gurskaya *et al.* (1996). They reported that a 1000–5000 fold enrichment of rare cDNAs (equivalent to isolating mRNAs present at only a few copies per cell) can be obtained without the need for multiple hybridizations/subtractions. Instead of physical or chemical removal of the common sequences, a PCR-based suppression system is used (see figure 5).

In SSH, excess driver cDNA is added to two portions of the tester cDNA which have been ligated with different adaptors. A first round of hybridization serves to enrich differentially expressed genes and equalize rare and abundant messages. Equalization occurs since reannealing is more rapid for abundant molecules than for rarer molecules due to the second order kinetics of hybridization (James and Higgins 1985). The two primary hybridization mixes are then mixed together in the presence of excess driver and allowed to hybridize further. This step permits the annealing of single stranded complementary sequences which did not hybridize in the primary hybridization, and in doing so generates templates for PCR amplification. Although there are several possible combinations of the single stranded molecules present in the secondary hybridization mix, only one particular combination (differentially expressed in the tester cDNA composed of complimentary strands having different adaptors) can amplify exponentially.

Having obtained the final differential display, two options are available if cloning of cDNAs is desired. One is to transform the whole of the final PCR reaction into competent cells. Transformed colonies can then be isolated and their inserts characterized by sequencing, restriction analysis or PCR. Alternatively, the final PCR products can be resolved on a gel and the individual bands excised, reamplified and cloned. The first approach is technically simpler and less time consuming. However, ligation/transformation reactions are known to be biased towards the cloning of smaller molecules, and so the final population of clones will probably not contain a representative selection of the larger products. In addition, although equalization theoretically occurs, observations in this laboratory suggest that this is by no means perfectly accomplished. Consequently, some gene species are present in a higher number than others and this will be represented in the final population of clones. Thus, in order to obtain a substantial proportion of those gene species that actually demonstrate differential expression in the tester population, the number of clones that will have to be screened after this step may be substantial. The second approach is initially more time consuming and technically demanding. However, it would appear to offer better prospects for cloning larger and low abundance gel products. In addition, one can incorporate a screening step that differentiates different products of different sequences but of the same size (HA-staining, see later). In this way, a good idea of the final number of clones to be isolated and identified can be achieved.

An alternative (or even complementary) approach is to use the final differential display reaction to screen a cDNA library to isolate full length clones for further characterization, or a DNA array (see later) to quickly identify known genes. SSH has been used in this laboratory t begin characterization of the short-term gene expression profiles of enzyme-inducers such as phenobarbital (Rockett *et al.* 1997) and Wy-14,643 (Rockett *et al.* unpublished observations). The isolation of differentially expressed genes in this manner enables the construction of a fingerprint

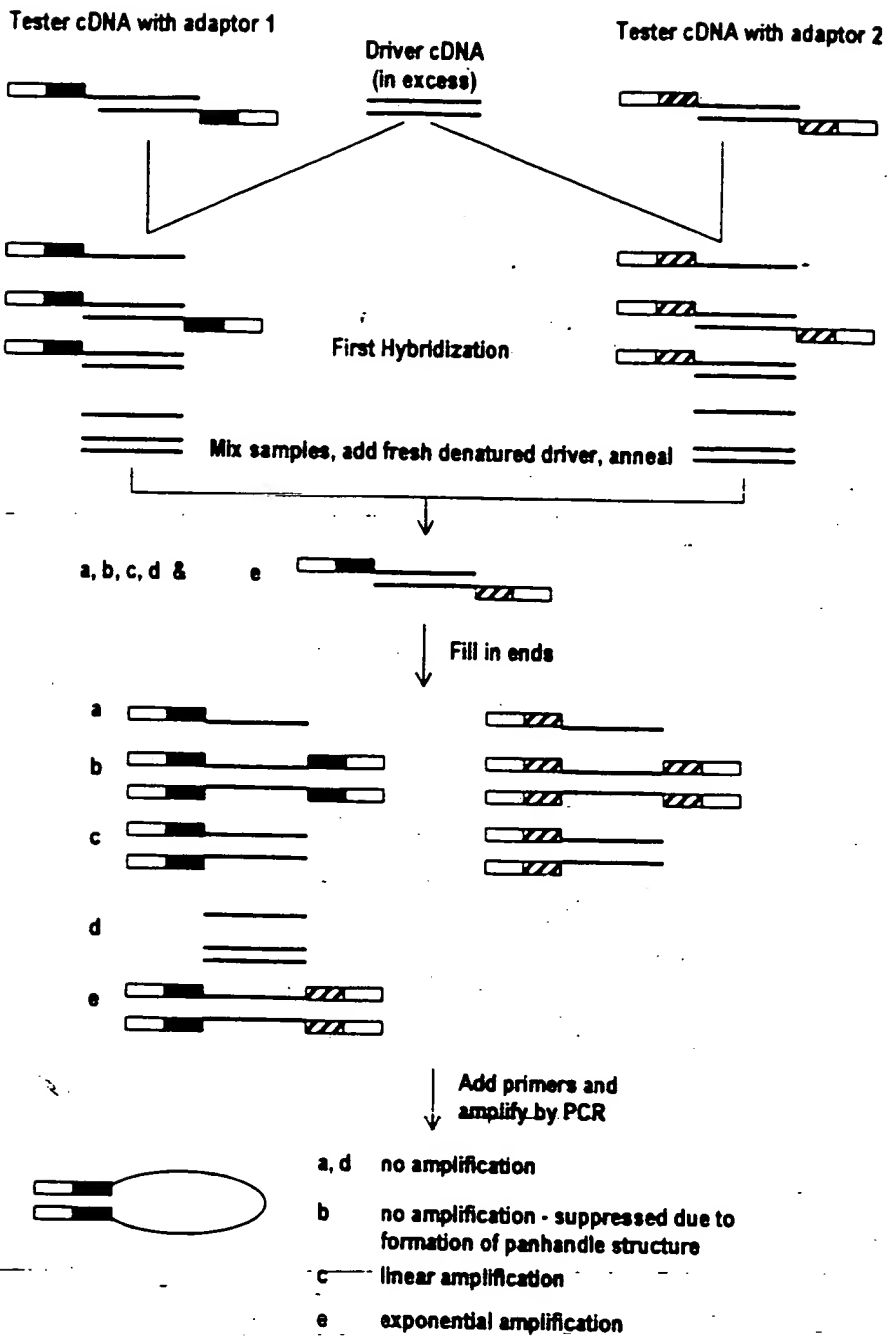

Figure 5. PCR-select cDNA subtraction. In the primary hybridization, an excess of driver cDNA is added to each tester cDNA population. The samples are heat denatured and allowed to hybridize for between 3 and 8 h. This serves two purposes: (1) to equalize rare and abundant molecules; and (2) to enrich for differentially expressed sequences—cDNAs that are not differentially expressed form type c molecules with the driver. In the secondary hybridization, the two primary hybridizations are mixed together without denaturing. Fresh denatured driver can also be added at this point to allow further enrichment of differentially expressed sequences. Type e molecules are formed in this secondary hybridization which are subsequently amplified using two rounds of PCR. The final products can be visualized on an agarose gel, labelled directly or cloned into a vector for downstream manipulation. As described by Diatchenko *et al.* (1996) and Guraskaya *et al.* (1996), with permission.

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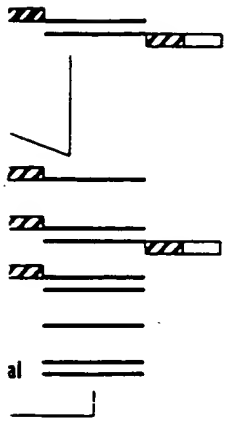

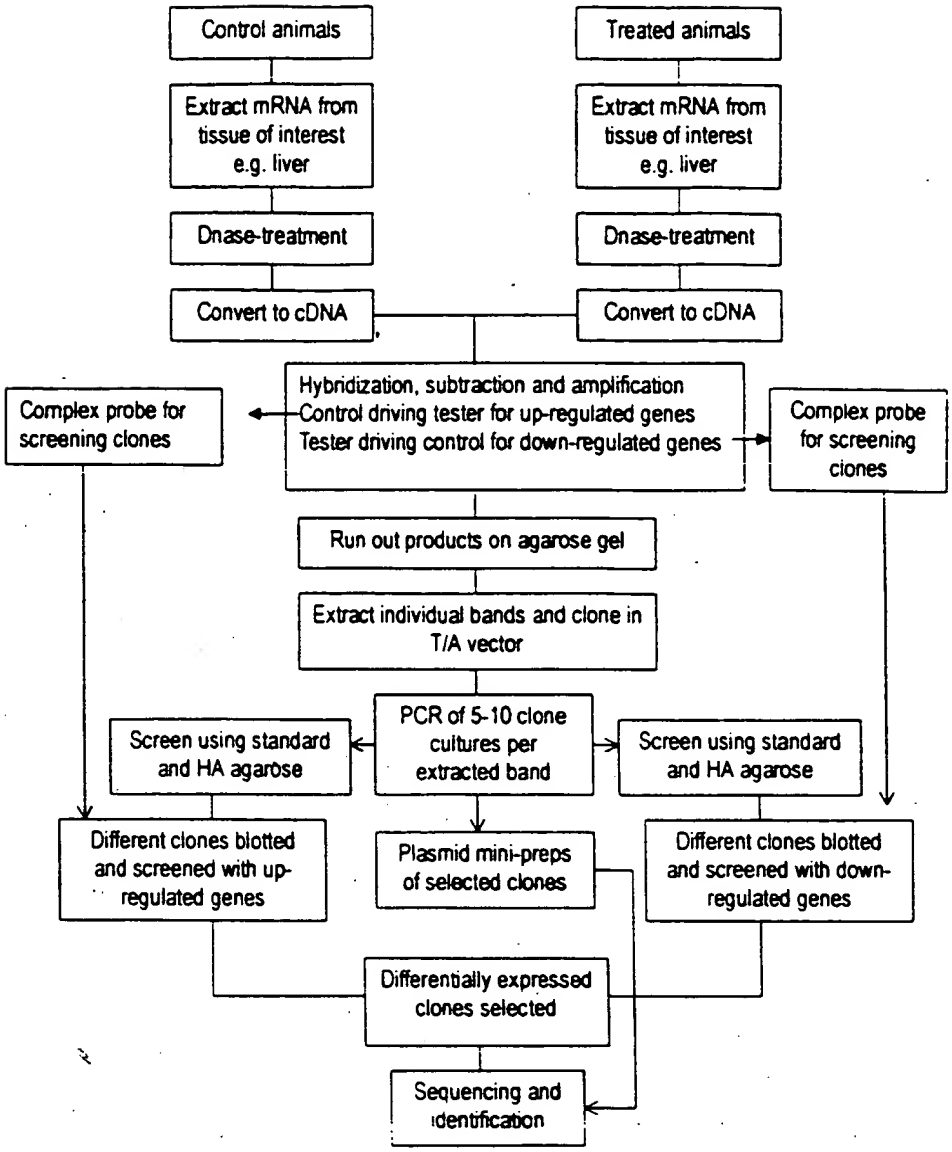

Figure 6. Flow diagram showing method used in this laboratory to isolate and identify clones of genes which are differentially expressed in rat liver following short term exposure to the enzyme inducers, phenobarbital and Wy-14,643.

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of expressed genes which are unique to each compound and time/dose point. Such information could be useful in short-term characterization of the toxic potential of new compounds by comparing the gene-expression profiles they elicit with those produced by known inducers. Figure 6 shows a flow diagram of the method used to isolate, verify and clone differentially expressed genes, and figure 7 shows expression profiles obtained from a typical SSH experiment. Subsequent sub-cloning of the individual bands, sequencing and gene data base interrogation reveals many genes which are either up- or down-regulated by phenobarbital in the rat (tables 2 and 3).

One of the advantages in using the SSH approach is that no prior knowledge is required of which specific genes are up/down-regulated subsequent to xenobiotic

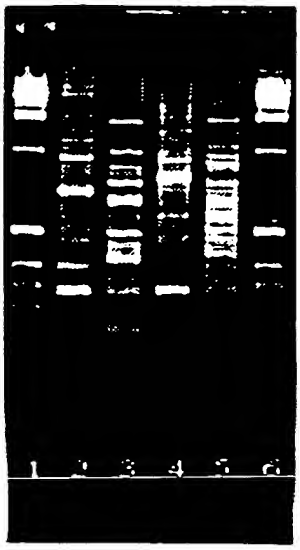

Figure 7. SSH display patterns obtained from rat liver following 3-day treatment with WY-14,643 or phenobarbital. mRNA extracted from control and treated livers was used to generate the differential displays using the PCR-Select cDNA subtraction kit (Clontech). Lane: 1—1kb ladder; 2—genes upregulated following Wy,14–643 treatment; 3—genes downregulated following Wy,14–643 treatment; 4—genes upregulated following phenobarbital treatment; 5—genes downregulated following phenobarbital treatment; 6—1kb ladder. Reproduced from Rockett *et al.* (1997), with permission.

exposure, and an almost complete complement of genes are obtained. For example, the peroxisome proliferator and non-genotoxic hepatocarcinogen Wy,14,643, up-regulates at least 28 genes and down-regulates at least 15 in the rat (a sensitive species) and produces 48 up- and 37 down-regulated genes in the guinea pig, a resistant species (Rockett, Swales, Esda and Gibson, unpublished observations). One of these genes, CD81, was up-regulated in the rat and down-regulated in the guinea pig following Wy-14,643 treatment. CD81 (alternatively named TAPA-1) is a widely expressed cell surface protein which is involved in a large number of cellular processes including adhesion, activation, proliferation and differentiation (Levy *et al.* 1998). Since all of these functions are altered to some extent in the phenomena of hepatomegaly and non-genotoxic hepatocarcinogenesis, it is intriguing, and probably mechanistically-relevant, that CD81 expression is differentially regulated in a resistant and susceptible species. However, the down-side of this approach is that the majority of genes can be sequenced and matched to database sequences, but the latter are predominantly expressed sequence tags or genes of completely unknown function, thus partially obscuring a realistic overall assessment of the critical genes of genuine biological interest. Notwithstanding the lack of complete funtional identification of altered gene expression, such gene profiling studies essentially provides a 'molecular fingerprint' in response to xenobiotic challenge, thereby serving as a mechanistically-relevant platform for further detailed investigations.

Differ ntial Display (DD)

Originally described as 'RNA fingerprinting by arbitrarily primed PCR' (Liang and Pardee 1992) this method is now more c mmonly referred to as 'differential

Table 2. Genes up-regulated in rat liver following 3-day exposure to phenobarbital.

Band number (approximate size in bp)	Highest sequence similarity	FASTA-EMBL gene identification
5 (1300)	93.5%	CYP2B1
7 (1000)	95.1%	Preproalbumin Serum albumin mRNA
8 (950)	98.3%	NCI-CGAP-Pr1 *H. sapiens* (EST)
10 (850)	95.7%	CYP2B1
11 (800)	Clone 1 94.9%	CYP2B1
	Clone 2 75.3%	CYP2B2
12 (750)	93.8%	TRPM-2 mRNA Sulfated glycoprotein
15 (600)	92.9%	Preproalbumin Serum albumin mRNA
16 (55)	Clone 1 95.2%	CYP2B1
	Clone 2 93.6%	Haptoglobulin mRNA partial alpha
21 (350)	99.3%	18S, 5.8S & 28S rRNa

Bands 1–4, 6, 9, 13, 14, and 17–20 are shown to be false positives by dot blot anaylsis and, therefore, are not sequenced. Derived from Rockett *et al.* (1997). It should be noted that the above genes do not represent the complete spectrum of genes which are up-regulated in rat liver by phenobarbital, but simply represents the genes sequenced and identified to date.

Table 3. Genes down-regulated in rat liver following 3-day exposure to phenobarbital.

Band number (approximate size in bp)	Highest sequence similarity	FASTA-EMBL gene identification
1 (1500)	95.3%	3-oxoacyl-CoA thiolase
2 (1200)	92.3%	Hemopoxin mRNA
3 (1000)	91.7%	Alpha-2u-globulin mRNA
7 (700)	Clone 1 77.2%	*M.musculus* Cl inhibitor
	Clone 2 94.5%	Electron transfer flavoprotein
	Clone 3 91.0%	*M. musculus* Topoisomerase 1 (Topo 1)
8 (650)	Clone 1 86.9%	Soares 2NbMT *M. musculus* (EST)
	Clone 2 96.2%	Alpha-2u-globulin (s-type) mRNA
9 (600)	Clone 1 86.9%	Soares mouse NML *M. musculus* (EST)
	Clone 2 82.0%	Soares p3NMF 19.5 *M. musculus* (EST)
10 (550)	73.8%	Soares mouse NML *M. musculus* (EST)
11 (525)	95.7%	NCI-CGAP-Pr1 *H. sapiens* (EST)
12 (375)	100.0%	Ribosomal protein
13 (23)	Clone 1 97.2%	Soares mouse embryo NbME135 (EST)
	Clone 2 100.0%	Fibrinogen B-beta-chain
	Clone 3 100.0%	Apolipoprotein E gene
14 (170)	96.0%	Soares p3NMF19.5 *M. musculus* (EST)
15 (140)	97.3%	Stratagene mouse testis (EST)
Others: (300)	96.7%	*R. norvegicus* RASP 1 mRNA
(275)	93.1%	Soares mouse mammary gland (EST)

EST = Expressed sequence tag. Bands 4–6 were shown to be false positives by dot blot analysis and, therefore, were not sequenced. Derived from Rockett *et al.* (1997). It should be noted that the above genes do not represent the complete spectrum of genes which are down-regulated in rat liver by phenobarbital, but simply represents the genes sequenced and identified to date.

display' (DD). In this method, all the mRNA species in the control and treated cell populations are amplified in separate reactions using revers transcriptase-PCR (RT-PCR). The products are then run side-by-sid on sequencing gels. Thos bands which are present in one display only, or which are much more intense in one

display compared to the other, are differentially expressed and may be recovered for further characterization. One advantage of this system is the speed with which it can be carried out—2 days to obtain a display and as little as a week to make and identify clones.

Two commonly used variations are based on different methods of priming the reverse transcription step (figure 8). One is to use an oligo dT with a 2-base 'anchor' at the 3'-end, e.g. 5' (dT_{11})CA 3' (Liang and Pardee 1992). Alternatively, an arbitrary primer may be used for 1st strand cDNA synthesis (Welsh *et al.* 1992). This variant of RNA fingerprinting has also been called 'RAP' (RNA Arbitrarily Primed)-PCR. One advantage of this second approach is that PCR products may be derived from anywhere in the RNA, including open reading frames. In addition, it can be used for mRNAs that are not polyadenylated, such as many bacterial mRNAs (Wong and McClelland 1994). In both cases, following reverse transcription and denaturation, second strand cDNA synthesis is carried out with an arbitrary primer (*arbitrary* primers have a single base at each position, as compared to *random* primers, which contain a mixture of all four bases at each position). The resulting PCR, thus, produces a series of products which, depending on the system (primer length and composition, polymerase and gel system), usually includes 50–100 products per primer set (Band and Sager 1989). When a combination of different dT-anchors and arbitrary primers are used, almost all mRNA species from a cell can be amplified. When the cDNA products from two different populations are analysed side by side on a polyacrylamide gel, differences in expression can be identified and the appropriate bands recovered for cloning and further analysis.

Although DD is perhaps the most popular approach used today for identifying differentially expressed genes, it does suffer from several perceived disadvantages:

(1) It may have a strong bias towards high copy number mRNAs (Bertioli *et al.* 1995), although this has been disputed (Wan *et al.* 1996) and the isolation of very low abundance genes may be achieved in certain circumstances (Guimeraes *et al.* 1995a).
(2) The cDNAs obtained often only represent the extreme 3' end of the mRNA (often the 3'-untranslated region), although this may not always be the case (Guimeraes *et al.* 1995a). Since the 3' end is often not included in Genbank and shows variation between organisms, cDNAs identified by DD cannot always be matched with their genes, even if they have been identified.
(3) The pattern of differential expression seen on the display often cannot be reproduced on Northern blots, with false positives arising in up to 70% of cases (Sun *et al.* 1994). Some adaptations have been shown to reduce false positives, including the use of two reverse transcriptases (Sung and Denman 1997), comparison of uninduced and induced cells over a time course (Burn *et al.* 1994) and comparison of DDPCR-products from two uninduced and two induced lines (Sompayrac *et al.* 1995). The latter authors also reported that the us of cytoplasmic RNA rather then total RNA reduces false positives arising from nuclear RNA that is not transported to the cytoplasm.

Further details of the background, strengths and weaknesses of the DD technique can be obtained from a review by McClelland *et al.* (1996) and from articles by Liang *et al.* (1995) and Wan *et al.* (1996).

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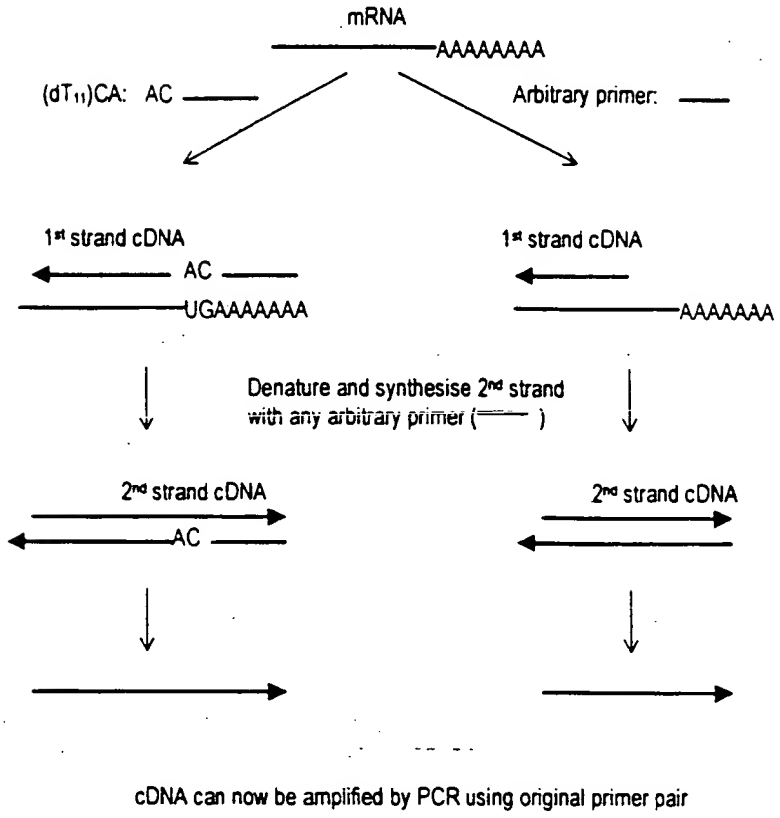

Figure 8. Two approaches to differential display (DD) analysis. 1st strand synthesis can be carried out either with a polydT_{11}NN primer (where N = G, C or A) or with an arbitrary primer. The use of different combinations of G, C and A to anchor the first strand polydT primer enables the priming of the majority of polyadenylated mRNAs. Arbitrary primers may hybridize at none, one or more places along the length of the mRNA, allowing 1st strand cDNA synthesis to occur at none, one or more points in the same gene. In both cases, 2nd strand synthesis is carried out with an arbitrary primer. Since these arbitrary primers for the 2nd strand may also hybridize to the 1st strand cDNA in a number of different places, several different 2nd strand products may be obtained from one binding point of the 1st strand primer. Following 2nd strand synthesis, the original set of primers is used to amplify the second strand products, with the result that numerous gene sequences are amplified.

Restriction endonuclease-facilitated analysis of gene expression

Serial Analysis of Gene Expression (SAGE)

A more recent development in the field of differential display is SAGE analysis (Velculescu *et al.* 1995). This method uses a different approach to those discussed so far and is based on two principles. Firstly, in more than 95% of cases, short nucleotide sequences ('tags') of only nine or 10 base pairs provide suffici nt information to identify their gene of origin. Secondly, concatonation (linking together in a series) of these tags allows sequencing of multiple cDNAs within a single clone. Figure 9 shows a schematic repres ntation of the SAGE process. In this pr cedure, doubl stranded cDNA from the test cells is synthesized with a biotinylated polydT primer. Following digesti n with a commonly cutting (4bp rec gnition sequence) restriction enzyme ('anchoring enzyme'), the 3′ ends of the cDNA population are captured with streptavidin beads. The captured population is

split into two and different adaptors ligated to the 5′ ends of each group. Incorporated into the adaptors is a recognition sequence for a type IIS restriction enzyme—one which cuts DNA at a defined distance (< 20 bp) from its recognition sequence. Hence, following digestion of each captured cDNA population with the IIS enzyme, the adaptors plus a short piece of the captured cDNA are released. The two populations are then ligated and the products amplified. The amplified products are cleaved with the original anchoring enzyme, religated (concatomers are formed in the process) and cloned. The advantage of this system is that hundreds of gene tags can be identified by sequencing only a few clones. Furthermore, the number of times a given transcript is identified is a quantitative measurement of that gene's abundance in the original population, a feature which facilitates identification of differentially expressed genes in different cell populations.

Some disadvantages of SAGE analysis include the technical difficulty of the method, a large amount of accurate sequencing is required, biased towards abundant mRNAs, has not been validated in the pharmaco/toxicogenomic setting and has only been used to examine well known tissue differences to date.

Gene Expression Fingerprinting (GEF)

A different capture/restriction digest approach for isolating differentially expressed genes has been described by Ivanova and Belyavsky (1995). In this method, RNA is converted to cDNA using biotinylated oligo(dT) primers. The cDNA population is then digested with a specific endonuclease and captured with magnetic streptavidin microbeads to facilitate removal of the unwanted 5′ digestion products. The use of restricted 3′-ends alone serves to reduce the complexity of the cDNA fragment pool and helps to ensure that each RNA species is represented by not more than one restriction product. An adaptor is ligated to facilitate subsequent amplification of the captured population. PCR is carried out with one adaptor-specific and one biotinylated polydT primer. The reamplified population is recaptured and the non-biotinylated strands removed by alkaline dissociation. The non-biotinylated strand is then resynthesized using a different adaptor-specific primer in the presence of a radiolabelled dNTP. The labelled immobilized 3′ cDNA ends are next sequentially treated with a series of different restriction endonucleases and the products from each digestion analysed by PAGE. The result is a fingerprint composed of a number of ladders (equal to the number of sequential digests used). By comparing test versus control fingerprints, it is possible to identify differentially expressed products which can then be isolated from the gel and cloned. The advantages of this procedure are that it is very robust and reproducible, and the authors estimate that 80–93% of cDNA molecules are involved in the final fingerprint. The disadvantage is that polyacrylamide gels can rarely resolve more than 300–400 bands, which compares poorly to the 1000 or more which are estimated to be produced in an average experiment. The use of 2-D gels such as those described by Uitterlinden *et al.* (1989) and Hatada *et al.* (1991) may help to overcome this problem.

A similar method for displaying restriction endonuclease fragments was later described by Prashar and Weissman (1996). However, instead of sequential digestion of the immobolized 3'-terminal cDNA fragments, these authors simply compared the profiles of the control and treated populations without further manipulation.

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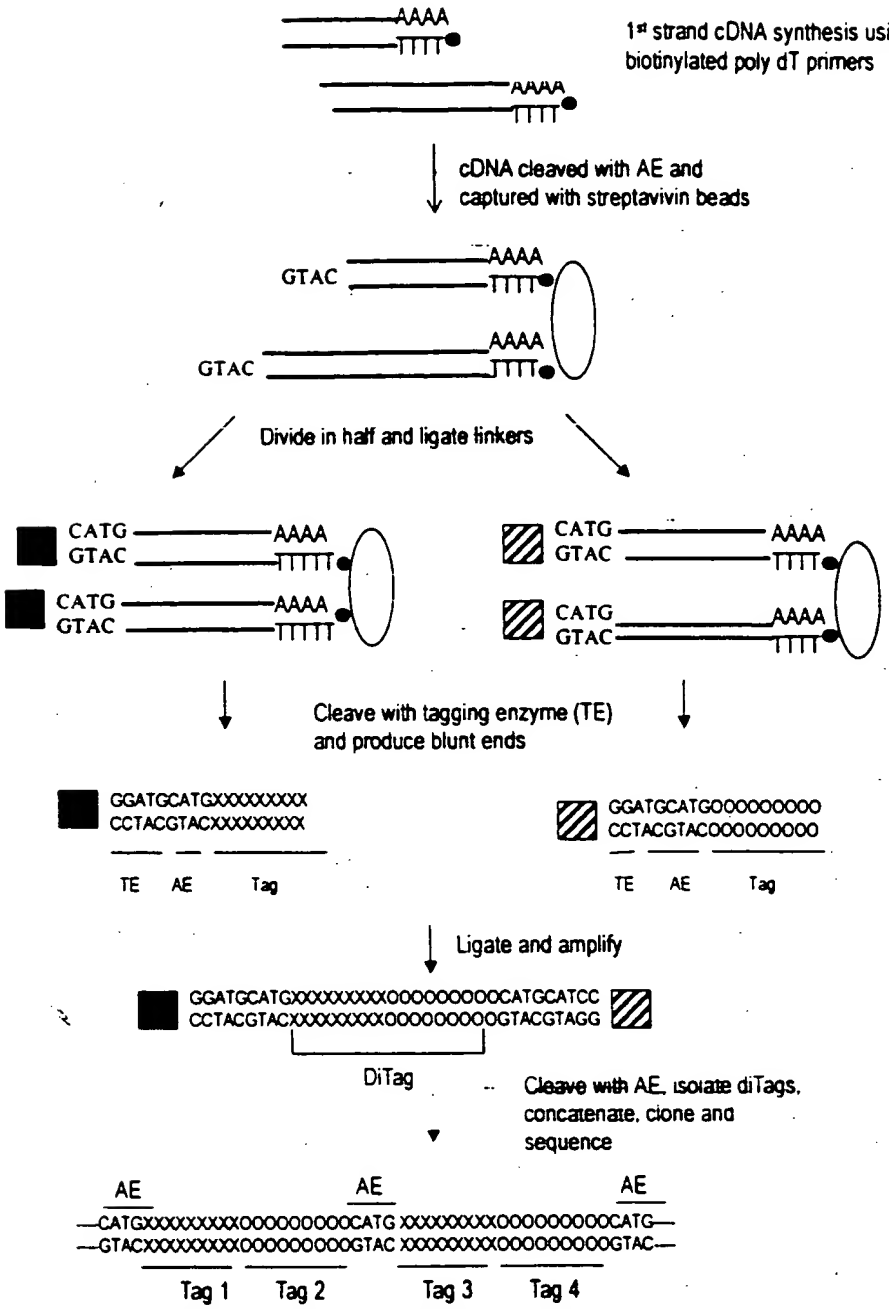

Figure 9. Serial analysis of gene expression (SAGE) analysis. cDNA is cleaved with an anchoring enzyme (AE) and the 3′ ends captured using streptavidin beads. The cDNA pool is divided in half and each portion ligated to a different linker, each containing a type IIS restriction site (tagging enzyme, TE). Restriction with the type IIS enzyme releases the linker plus a short length of cDNA (XXXXX and OOOOO indicate nucleotides of different tags). The two pools of tags are then ligated and amplified using linker-specific primers. Following PCR, the products are cleaved with the AE and the ditags isolated from the linkers using PAGE. The ditags are then ligated (during which process, concatenization occurs) and cloned into a vector of choice for sequencing. After Velculescu *et al.* (1995), with permission.

DNA arrays

'Open' differential display systems are cumbersome in that it takes a great deal of time to extract and identify candidate genes and then confirm that they are indeed up- or down-regulated in the treated compared to the control tissue. Normally, the latter process is carried out using Northern blotting or RT-PCR. Even so, each of the aforementioned steps produce a bottleneck to the ultimate goal of rapid analysis of gene expression. These problems will likely be addressed by the development of so-called DNA arrays (e.g. Gress *et al.* 1992, Zhao *et al.* 1995, Schena *et al.* 1996), the introduction of which has signalled the next era in differential gene expression analysis. DNA arrays consist of a gridded membrane or glass 'chips' containing hundreds or thousands of DNA spots, each consisting of multiple copies of part of a known gene. The genes are often selected based on previously proven involvement in oncogenesis, cell cycling, DNA repair, development and other cellular processes. They are usually chosen to be as specific as possible for each gene and animal species. Human and mouse arrays are already commercially available and a few companies will construct a personalized array to order, for example Clontech Laboratories and Research Genetics Inc. The technique is rapid in that hundreds or even thousands of genes can be spotted on a single array, and that mRNA/cDNA from the test populations can be labelled and used directly as probe. When analysed with appropriate hardware and software, arrays offer a rapid and quantitative means to assess differences in gene expression between two cell populations. Of course, there can only be identification and quantitation of those genes which are in the array (hence the term 'closed' system). Therefore, one approach to elucidating the molecular mechanisms involved in a particular disease/development system may be to combine an open and closed system—a DNA array to directly identify and quantitate the expression of known genes in mRNA populations, and an open system such as SSH to isolate unknown genes which are differentially expressed.

One of the main advantages of DNA arrays is the huge number of gene fragments which can be put on a membrane—some companies have reported gridding up to 60000 spots on a single glass 'chip' (microscope slide). These high density chip-based micro-arrays will probably become available as mass-produced off-the-shelf items in the near future. This should facilitate the more rapid determination of differential expression in time and dose-response experiments. Aside from their high cost and the technical complexities involved in producing and probing DNA arrays, the main problem which remains, especially with the newer micro-array (gene-chip) technologies, is that results are often not wholly reproducible between arrays. However, this problem is being addressed and should be resolved within the next few years.

EST databases as a means to identify differentially expressed genes

Expressed sequence tags (ESTs) are partial sequences of clones obtained from cDNA libraries. Even though most ESTs have no formal identity (putative identification is the best to be hoped for), they have proven to be a rapid and efficient means of discovering new genes and can be used to generate profiles of gene-expression in specific cells. Since they were first described by Adams *et al.* (1991), there has been a huge explosion in EST production and it is estimated that there are now well over a million such sequences in the public domain, representing over half

of all human genes (Hillier *et al.* 1996). This large number of freely available sequences (both sequence information and clones are normally available royalty-free from the originators) has enabled the development of a new approach towards differential gene expression analysis as described by Vasmatzis *et al.* (1998). The approach is simple in theory: EST databases are first searched for genes that have a number of related EST sequences from the target tissue of choice, but none or few from non-target tissue libraries. Programmes to assist in the assembly of such sets of overlapping data may be developed in-house or obtained privately or from the internet. For example, the Institute for Genomic Research (TIGR, found at http://www.tigr.org) provides many software tools free of charge to the scientific community. Included amongst these is the TIGR assembler (Sutton *et al.* 1995), a tool for the assembly of large sets of overlapping data such as ESTs, bacterial artificial chromosomes (BAC)s, or small genomes. Candidate EST clones representing different genes are then analysed using RNA blot methods for size and tissue specificity and, if required, used as probes to isolate and identify the full length cDNA clone for further characterization. In practice however, the method is rather more involved, requiring bioinformatic and computer analysis coupled with confirmatory molecular studies. Vasmatzis *et al.* (1998) have described several problems in this fledgling approach, such as separating highly homologous sequences derived from different genes and an overemphasis of specificity for some EST sequences. However, since these problems will largely be addressed by the development of more suitable computer algorithms and an increased completeness of the EST database, it is likely that this approach to identifying differentially expressed genes may enjoy more patronage in the future.

Problems and potential of differential expression techniques

The holistic or single cell approach?

When working with *in vivo* models of differential expression, one of the first issues to consider must be the presence of multiple cell types in any given specimen. For example, a liver sample is likely to contain not only hepatocytes, but also (potentially) Ito cells, bile ductule cells, endothelial cells, various immune cells (e.g. lymphocytes, macrophages and Kupffer cells) and fibroblasts. Other tissues will each have their own distinctive cell populations. Also, in the case of neoplastic tissue, there are almost always normal, hyperplastic and/or dysplastic cells present in a sample. One must, therefore, be aware that genes obtained from a differential display experiment performed on an animal tissue model may not necessarily arise exclusively from the intended 'target' cells, e.g. hepatocytes/neoplastic cells. If appropriate, further analyses using immunohistochemistry, *in situ* hybridization or *in situ* RT-PCR should be used to confirm which cell types are expressing the gene(s) of interest. This problem is probably most acute for those studying the differential expression of genes in the development of different cell types, where there is a need to examine homologous cell populations. The problem is now being addressed at the National Cancer Institute (Bethesda, MD, USA) where new microdisection techniques have been employed to assist in their gene analysis programme, the Cancer Genome Anatomy Project (CGAP) (For more information se web site: http://www.ncbi.nlm.nih.gov/ncicgap/intro.html). There are also separation techniques available that utilise cell-specific antigens as a means to isolate target cells,

e.g. fluorescence activated cell sorting (FACS) (Dunbar *et al.* 1998, Kas-Deelen *et al.* 1998) and magnetic bead technology (Richard *et al.* 1998, Rogler *et al.* 1998).

However, those taking a holistic approach may consider this issue unimportant. There is an equally appropriate view that all those genes showing altered expression within a compromized tissue should be taken into consideration. After all, since all tissues are complex mixes of different, interacting cell types which intimately regulate each other's growth and development, it is clear that each cell type could in some way contribute (positively or negatively) towards the molecular mechanisms which lie behind responses to external stimuli or neoplastic growth. It is perhaps then more informative to carry out differential display experiments using *in vivo* as opposed to *in vitro* models, where uniform populations of identical cells probably represent a partial, skewed or even inaccurate picture of the molecular changes that occur.

The incidence and possible implications of inter-individual biological variation should be considered in any approach where whole animal models are being used. It is clear that individuals (humans and animals) respond in different ways to identical stimuli. One of the best characterized examples is the debrisoquine oxidation polymorphism, which is mediated by cytochrome CYP2D6 and determines the pharmacokinetics of many commonly prescribed drugs (Lennard 1993, Meyer and Zanger 1997). The reasons for such differences are varied and complex, but allelic variations, regulatory region polymorphisms and even physical and mental health can all contribute to observed differences in individual responses. Careful thought should, therefore, be given to the specific objectives of the study and to the possible value of pooling starting material (tissue/mRNA). The effect of this can be beneficial through the ironing out of exaggerated responses and unimportant minor fluctuations of (mechanistically) irrelevant genes in individual animals, thus providing a clearer overall picture of the general molecular mechanisms of the response. However, at the same time such minor variations may be of utmost importance in deciding the ability of individual animals to succumb to or resist the effects of a given chemical/disease.

How efficient are differential expression techniques at recovering a high percentage of differentially expressed genes?

A number of groups have produced experimental data suggesting that mammalian cells produce between 8000–15000 different mRNA species at any on time (Mechler and Rabbitts 1981, Hedrick *et al.* 1984, Bravo 1990), although figures as high as 20–30000 have also been quoted (Axel *et al.* 1976). Hedrick *et al.* (1984) provided evidence suggesting that the majority of these belong to the rare abundanc class. A breakdown of this abundance distribution is shown in table 1.

When the results of differential display experiments have been compared with data obtained previously using other methods, it is apparent that not all differentially expressed mRNAs are represented in the final display. In particular, rare messages (which, importantly, often include regulatory proteins) are not easily recov red using differential display systems. This is a major shortcoming, as the majority of mRNA species exist at levels of less than 0.005 % of the total population (table 1). Bertioli *et al.* (1995) examined the effici ncy of DD templates (heterogeneous mRNA populations) for recovering rare messages and were unable to detect mRNA

species present at less than 1.2% of the total mRNA population—equivalent to an intermediate or abundant species. Interestingly, when simple model systems (single target only) were used instead of a heterogeneous mRNA population, the same primers could detect levels of target mRNA down to 10000 × smaller. These results are probably best explained by competition for substrates from the many PCR products produced in a DD reaction.

The numbers of differentially expressed mRNAs reported in the literature using various model systems provides further evidence that many differentially expressed mRNAs are not recovered. For example, DeRisi *et al.* (1997) used DNA array technology to examine gene expression in yeast following exhaustion of sugar in the medium, and found that more than 1700 genes showed a change in expression of at least 2-fold. In light of such a finding, it would not be unreasonable to suggest that of the 8000–15 000 different mRNA species produced by any given mammalian cell, up to 1000 or more may show altered expression following chemical stimulation. Whilst this may be an extreme figure, it is known that at least 100 genes are activated/upregulated in Jurkat (T-) cells following IL-2 stimulation (Ullman *et al.* 1990). In addition, Wan *et al.* (1996) estimated that interferon-γ-stimulated HeLa cells differentially express up to 433 genes (assuming 24000 distinct mRNAs expressed by the cells). However, there have been few publications documenting anywhere near the recovery of these numbers. For example, in using DD to compare normal and regenerating mouse liver, Bauer *et al.* (1993) found only 70 of 38000 total bands to be different. Of these, 50% (35 genes) were shown to correspond to differentially expressed bands. Chen *et al.* (1996) reported 10 genes upregulated in female rat liver following ethinyl estradiol treatment. McKenzie and Drake (1997) identified 14 different gene products whose expression was altered by phorbol myristate acetate (PMA, a tumour promoter agent) stimulation of a human myelomonocytic cell line. Kilty and Vickers (1997) identified 10 different gene products whose expression was upregulated in the peripheral blood leukocytes of allergic disease sufferers. Linskens *et al.* (1995) found 23 genes differentially expressed between young and senescent fibroblasts. Techniques other than DD have also provided an apparent paucity of differentially expressed genes. Using SH for example, Cao *et al.* (1997) found 15 genes differentially expressed in colorectal cancer compared to normal mucosal epithelium. Fitzpatrick *et al.* (1995) isolated 17 genes upregulated in rat liver following treatment with the peroxisome proliferator, clofibrate; Philips *et al.* (1990) isolated 12 cDNA clones which were upregulated in highly metastatic mammary adenocarcinoma cell lines compared to poorly metastatic ones. Prashar and Weissman (1996) used 3′ restriction fragment analysis and identified approximately 40 genes showing altered expression within 4 h of activation of Jurkat T-cells. Groenink and Leegwater (1996) analysed 27 gene fragments isolated using SSH of delayed early response phase of liver regeneration and found only 12 to be upregulated.

In the laboratory, SSH was used to isolate up to 70 candidate genes which appear to show altered expression in guinea pig liver following short-term treatment with the peroxisome proliferator, WY-14,643 (Rockett, Swales, Esdaile and Gibson, unpublished observations). However, these findings have still to be confirmed by analysis of the extracted tissue mRNA for differential expression of these sequences. Whilst the latest differential display technologies are purported to include design and experimental modifications to overcom this lack of efficiency (in both the total number of differentially expressed genes recovered and the percentage that are true

positives), it is still not clear if such adaptations are practically effective—proving efficiency by spiking with a known amount of limited numbers of artificial construct(s) is one thing, but isolating a high percentage of the rare messages already present in an mRNA population is another. Of course, some models will genuinely produce only a small number of differentially expressed genes. In addition, there are also technical problems that can reduce efficiency. For example, mRNAs may have an unusual primary structure that effectively prevents their amplification by PCR-based systems. In addition, it is known that under certain circumstances not all mRNAs have 3′ polyA sites. For example, during *Xenopus* development, deadenylation is used as a means to stabilize RNAs (Voeltz and Steitz 1998), whilst preferential deadenylation may play a role in regulating Hsp70 (and perhaps, therefore, other stress protein) expression in *Drosophila* (Dellavalle *et al.* 1994). The presence of deadenylated mRNAs would clearly reduce the efficiency of systems utilizing a polydT reverse transcription step. The efficiency of any system also depends on the quality of the starting material. All differential display t chniques use mRNA as their target material. However, it is difficult to isolate mRNA that is completely free of ribosomal RNA. Even if polydT primers are used to prime first strand cDNA synthesis, ribosomal RNA is often transcribed to some degree (Clontech PCR-Select cDNA Subtraction kit user manual). It has been shown, at least in the case of SSH, that a high rRNA:mRNA ratio can lead to inefficient subtractive hybridization (Clontech PCR-Select cDNA Subtraction kit user manual), and there is no reason to suppose that it will not do likewise in other SH approaches. Finally, those techniques that utilise a presubtraction amplification step (e.g. RDA) may present a skewed representation since some sequences amplify better than others.

Of course, probably the most important consideration is the temporal factor. It is clear that any given differential display experiment can only interrogate a cell at one point in time. It may well be that a high percentage of the genes showing altered expression at that time are obtained. However, given that disease processes and responses to environmental stimuli involve dynamic cascades of signalling, regulation, production and action, it is clear that all those genes which are switched on/off at different times will not be recovered and, therefore, vital information may well be missed. It is, therefore, imperative to obtain as much information about the model system beforehand as possible, from which a strategy can be derived for targeting specific time points or events that are of particular interest to the investigator. One way of getting round this problem of single time point analysis is to conduct the experiment over a suitable time course which, of course, adds substantially to the amount of work involved.

How sensitive are differential expression technologies?

There has been little published data that addresses the issue of how large th change in expression must be for it to permit isolation of the gene in question with the various differential expression technologies. Although the isolation of gen s whose expression is changed as little as 1.5-fold has been reported using SSH (Groenink and Leegwater 1996), it appears that thos demonstrating a change in excess of 5-fold are more likely to be picked up. Thus, ther is a 'grey zone' in between where small changes could fade in and out of isolation between

experiments and animals. DD, on the other hand, is not subject to this grey zone since, unlike SH approaches, it does not amplify the difference in expression between two samples. Wan *et al.* (1996) reported that differences in expression of twofold or more are detectable using DD.

Resolution and visualization of differential expression products

It seems highly improbable with current technology that a gel system could be developed that is able to resolve all gene species showing altered expression in any given test system (be it SH- or DD-based). Polyacrylamide gel electrophoresis (PAGE) can resolve size differences down to 0.2% (Sambrook *et al.* 1989) and are used as standard in DD experiments. Even so, it is clear that a complex series of gene products such as those seen in a DD will contain unresolvable components. Thus, what appears to be one band in a gel may in fact turn out to be several. Indeed, it has been well documented (Mathieu-Daude *et al.* 1996, Smith *et al.* 1997) that a single band extracted from a DD often represents a composite of heterogeneous products, and the same has been found for SSH displays in this laboratory (Rockett *et al.* 1997). One possible solution was offered by Mathieu-Daude *et al.* (1996), who extracted and reamplified candidate bands from a DD display and used single strand conformation polymorphism (SSCP) analysis to confirm which components represented the truly differentially expressed product.

Many scientists often try to avoid the use of PAGE where possible because it is technically more demanding than agarose gel electrophoresis (AGE). Unfortunately, high resolution agarose gels such as Metaphor (FMC, Lichfield, UK) and AquaPor HR (National Diagnostics, Hessle, UK), whilst easier to prepare and manipulate than PAGE, can only separate DNA sequences which differ in size by around 1.5–2% (15–20 base pairs for a 1Kb fragment). Thus, SSH, RDA or other such products which differ in size by less than this amount are normally not resolvable. However, a simple technique does in fact exist for increasing the resolving power of AGE—the inclusion of HA-red (10-phenyl neutral red-PEG ligand) or HA-yellow (bisbenzamide-PEG ligand) (Hanse Analytik GmbH, Bremen, Germany) in a gel separates identical or closely sized products on base content. Specifically, HA-red and -yellow selectively bind to GC and AT DNA motifs, respectively (Wawer *et al.* 1995, Hanse Analytik 1997, personal communication). Since both HA-stains possess an overall positive charge, they migrate towards the cathode when an electric field is applied. This is in direct opposition to DNA, which is negatively charged and, therefore, migrates towards the anode. Thus, if two DNA clones are identical in size (as perceived on a standard high resolution agarose gel), but differ in AT/GC content, inclusion of a HA-dye in the gel will effectively retard the migration of one of the sequences compared to the other, effectively making it apparently larger and, thus, providing a means of differentiating between the two. The use of HA-red has been shown to resolve sequences with an AT variation of less than 1% (Wawer *et al.* 1995), whilst Hans Analytik have reported that HA staining is so sensitive that in one case it was used to distinguish two 567bp sequences which differed by only a single point mutation (Hanse Analytik 1996, personal communication). Therefore, if one wishes to check whether all the clones produced from a specific band in a differential display experiment are derived from the same gene species, a small amount of reamplified or digested clone can be run on a standard high resolution gel, and a second aliquot

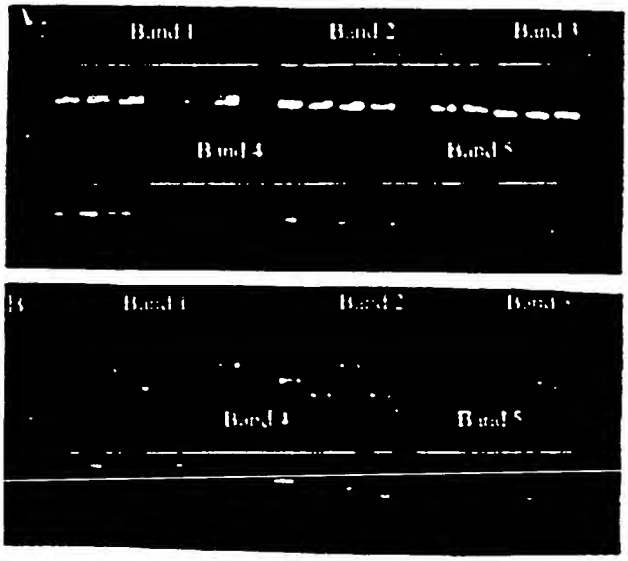

Figure 10. Discrimination of clones of identical/nearly identical size using HA-red. Bands of decreasing size (1–5) were extracted from the final display of a suppression subtractive hybridization experiment and cloned. Seven colonies were picked at random from each cloned band and their inserts amplified using PCR. The products were run on two gels, (A) a high resolution 2% agarose gel, and (B) a high resolution 2% agarose gel containing 1 U/ml HA-red. With few exceptions, all the clones from each band appear to be the same size (gel A). However, the presence of HA-red (gel B), which separates identically-sized DNA fragments based on the percentage of GC within the sequence, clearly indicates the presence of different gene species within each band. For example, even though all five re-amplified clones of band 1 appear to be the same size, at least four different gene species are represented.

in a similar gel containing one of the HA-stains. The standard gel should indicate any gross size differences, whilst the HA-stained gel should separate otherwise unresolvable species (on standard AGE) according to their base content. Geisinger *et al.* (1997) reported successful use of this approach for identifying DD-derived clones. Figure 10 shows such an experiment carried out in this laboratory on clones obtained from a band extracted from an SSH display.

An alternative approach is to carry out a 2-D analysis of the differential display products. In this approach, size-based separation is first carried out in a standard agarose gel. The gel slice containing the display is then extracted and incorporated in to a HA gel for resolution based on AT/GC content.

Of course, one should always consider the possibility of there being different gene species which are the same size and have the same GC/AT content. However, even these species are not unresolvable given some effort—again, one might use SSCP, or perhaps a denaturing gradient gel electrophoresis (DGGE) or temperature gradient field electrophoresis (TGGE) approach to resolve the contents of a band, either directly on the extracted band (Suzuki *et al.* 1991) or on the reamplifi d product.

The requirement of some differential display techniques to visualize large numbers of products (e.g. DD and GEF) can also present a problem in that, in terms of numbers, the resolution of PAGE rarely exceeds 300–400 bands. One approach to overcoming this might be to use 2-D gels such as those described by Uitterlinden *et al.* (1989) and Hatada *et al.* (1991).

Extraction of differentially expressed bands from a gel can be complex since, in some cases (e.g. DD, GEF), the results are visualized by autoradiographic means, such that precise overlay of the developed film on the gel must occur if the correct band is to be extracted for further analysis. Clearly, a misjudged extraction can account for many man-hours lost. This problem, and that of the use of radioisotopes, has been addressed by several groups. For example, Lohmann *et al.* (1995) demonstrated that silver staining can be used directly to visualize DD bands in horizontal PAGs. An *et al.* (1996) avoided the use of radioisotopes by transferring a small amount (20–30%) of the DNA from their DD to a nylon membrane, and visualizing the bands using chemiluminescent staining before going back to extract the remaining DNA from the gel. Chen and Peck (1996) went one step further and transferred the entire DD to a nylon membrane. The DNA bands were then visualized using a digoxigenin (DIG) system (DIG was attached to the polydT primers used in the differential display procedure). Differentially expressed bands were cut from the membrane and the DNA eluted by washing with PCR buffer prior to reamplification.

One of the advantages of using techniques such as SSH and RDA is that the final display can be run on an agarose gel and the bands visualized with simple ethidium bromide staining. Whilst this approach can provide acceptable results, overstaining with SYBR Green I or SYBR Gold nucleic acid stains (FMC) effectively enhances the intensity and sharpness of the bands. This greatly aids in their precise extraction and often reveals some faint products that may otherwise be overlooked. Whilst differential displays stained with SYBR Green I are better visualized using short wavelength UV (254 nm) rather than medium wavelength (306 nm), the shorter wavelength is much more DNA damaging. In practice, it takes only a few seconds to damage DNA extracted under 254 nm irradiation, effectively preventing reamplification and cloning. The best approach is to overstain with SYBR Green I and extract bands under a medium wavelength UV transillumination.

The possible use of 'microfingerprinting' to reduce complexity

Given the sheer number of gene products and the possible complexity of each band, an alternative approach to rapid characterization may be to use an enhanced analysis of a small section of a differential display—a 'sub-fingerprint' or 'micro-fingerprint'. In this case, one could concentrate on those bands which only appear in a particular chosen size region. Reducing the fingerprint in this way has at least two advantages. One is that it should be possible to use different gel types, concentrations and run times tailored exactly to that region. Currently, one might run products from 100–3000+ bp on the same gel, which leads to compromize in the gel system being used and consequently to suboptimal resolution, both in terms of size and numbers, and can lead to problems in the accurate excision of individual bands. Secondly, it may be possible to enhance resolution by using a 2-D analysis using a HA-stain, as described earlier. In summary, if a range of gene product sizes is carefully chosen to included certain 'relevant' genes, the 2-D system standardiz d, and appropriate gene analysis used, it may be possible to develop a method for the early and rapid identification of compounds which have similar or widely different cellular ffects. If the prognosis for xposure to one or more other chemicals which display a similar profile is already known, then one could perhaps predict similar effects for any new compounds which show a similar micro-fingerprint.

An alternative approach to microfingerprinting is to examine altered expression in specific families of genes through careful selection of PCR primers and/or post-reaction analysis. Stress genes, growth factors and/or their receptors, cell cycling genes, cytochromes P450 and regulatory proteins might be considered as candidates for analysis in this way. Indeed, some off-the-shelf DNA arrays (e.g. Clontech's Atlas cDNA Expression Array series) already anticipated this to some degree by grouping together genes involved in different responses e.g. apoptosis, stress, DNA-damage response etc.

Screening

False positives

The generation of false positives has been discussed at length amongst the differential display community (Liang *et al.* 1993, 1995, Nishio *et al.* 1994, Sun *et al.* 1994, Sompayrac *et al.* 1995). The reason for false positives varies with the technique being used. For instance, in RDA, the use of adaptors which have not been HPLC purified can lead to the production of false positives through illegitimate ligation events (O'Neill and Sinclair 1997), whilst in DD they can arise through PCR artifacts and illegitemate transcription of rRNA. In SH, false positives appear to be derived largely from abundant gene species, although some may arise from cDNA/mRNA species which do not undergo hybridization for technical reasons.

A quick screening of putative differentially expressed clones can be carried out using a simple dot blot approach, in which labelled first strand probes synthesized from tester and driver mRNA are hybridized to an array of said clones (Hedrick *et al.* 1984, Sakaguchi *et al.* 1986). Differentially expressed clones will hybridize to tester probe, but not driver. The disadvantage of this approach is that rare species may not generate detectable hybridization signals. One option for those using SSH is to screen the clones using a labelled probe generated from the subtracted cDNA from which it was derived, and with a probe made from the reverse subtraction reaction (ClonTechniques 1997a). Since the SSH method enriches rare sequences, it should be possible to confirm the presence of clones representing low abundance genes. Despite this quick screening step, there is still the need to go back to the original mRNA and confirm the altered expression using a more quantitative approach. Although this may be achieved using Northern blots, the sensitivity is poor by today's high standards and one must rely on PCR methods for accurate and sensitive determinations (see below).

Sequence analysis

The majority of differential display procedures produce final products which ar between 100 and 1000bp in size. However, this may considerably reduce the siz of the sequence for analysis of the DNA databases. This in turn leads to a reduced confidence in the result—several families of genes have members whose DNA sequences are almost identical except in a few key stretches, e.g. the cytochrome P450 gene superfamily (Nelson *et al.* 1996). Thus, does the clone identified as being almost identical to gene X_0 really come from that gene, or its brother gene X_1 or its as yet undiscovered sister X_2? For example, using SSH, part of a gene was isolated,

which was up-regulated in the liver of rats exposed to Wy-14,643 and was identified by a FASTA search as being transferrin (data not shown). However, transferrin is known to be downregulated by hypolipidemic peroxisome proliferators such as Wy-14,643 (Hertz *et al.* 1996), and this was confirmed with subsequent RT-PCR analysis. This suggests that the gene sequence isolated may belong to a gene which is closely related to transferrin, but is regulated by a different mechanism.

A further problem associated with SH technology is redundancy. In most cases before SH is carried out, the cDNA population must first be simplified by restriction digestion. This is important for at least two reasons:

(1) To reduce complexity—long cDNA fragments may form complex networks which prevent the formation of appropriate hybrids, especially at the high concentrations required for efficient hybridization.
(2) Cutting the cDNAs into small fragments provides better representation of individual genes. This is because genes derived from related but distinct members of gene families often have similar coding sequences that may cross-hybridize and be eliminated during the subtraction procedure (Ko 1990). Furthermore, different fragments from the same cDNA may differ considerably in terms of hybridization and amplification and, thus, may not efficiently do one or the other (Wang and Brown 1991). Thus, some fragments from differentially expressed cDNAs may be eliminated during subtractive hybridization procedures. However, other fragments may be enriched and isolated. As a consequence of this, some genes will be cut one or more times, giving rise to two or more fragments of different sizes. If those same genes are differentially expressed, then two or more of the different size fragments may come through as separate bands on the final differential display, increasing the observed redundancy and increasing the number of redundant sequencing reactions.

Sequence comparisons also throw up another important point—at what degree of sequence similarity does one accept a result. Is 90% identitiy between a gene derived from your model species and another acceptably close? Is 95% between your sequence and one from the same species also acceptable? This problem is particularly relevant when the forward and reverse sequence comparisons give similar sequences with completely different gene species! An arbitrary decision seems to be to allocate genes that are definite (95% and above similarity) and then group those between 60 and 95% as being related or possible homologues.

Quantitative analysis

At some point, one must give consideration to the quantitative analysis of the candidate genes, either as a means of confirming that they are truly differentially expressed, or in order to establish just what the differences are. Northern blot analysis is a popular approach as it is relatively easy and quick to perform. However, the major drawback with Northern blots is that they are often not sensitive enough to detect rare sequences. Since the majority of messages expressed in a cell are of low abundance (see table 1), this is a major problem. Consequently, RT-PCR may be th method of choice for confirming differential expression. Although the procedure is som what more complex than Northern analysis, requiring synthesis of primers and optimization of reaction conditions for each gene species, it is now possible to set up high throughput PCR systems using mulitchannel pipettes, 96+-well plates and

appropriate thermal cycling technology. Whilst quantitative analysis is more desirable, being more accurate and without reliance on an internal standard, the money and time needed to develop a competitor molecule is often excessive, especially when one might be examining tens or even hundreds of gene species. The use of semi-quantitative analysis is simpler, although still relatively involved. One must first of all choose an internal standard that does not change in the test cells compared to the controls. Numerous reference genes have been tried in the past, for example interferon-gamma (IFN-γ, Frye *et al.* 1989), β-actin (Heuval *et al.* 1994), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Wong *et al.* 1994), dihydrofolate reductase (DHFR, Mohler and Butler 1991), β-2-microglobulin (β-2-m, Murphy *et al.* 1990), hypoxanthine phosphoribosyl transferase (HPRT, Foss *et al.* 1998) and a number of others (ClonTechniques 1997b). Ideally, an internal standard should not change its level of expression in the cell regardless of cell age, stage in the cell cycle or through the effects of external stimuli. However, it has been shown on numerous occasions that the levels of most housekeeping genes currently used by the research community do in fact change under certain conditions and in different tissues (ClonTechniques 1997b). It is imperative, therefore, that preliminary experiments be carried out on a panel of housekeeping genes to establish their suitability for use in the model system.

Interpretation of quantitative data must also be treated with caution. By comparing the lists of genes identified by differential expression one can perhaps gain insight into why two different species react in different ways to external stimuli. For example, rats and mice appear sensitive to the non-genotoxic effects of a wide range of peroxisome proliferators whilst Syrian hamsters and guinea pigs are largely resistant (Orton *et al.* 1984, Rodricks and Turnbull 1987, Lake *et al.* 1989, 1993, Makowska *et al.* 1992). A simplified approach to resolving the reason(s) why is to compare lists of up- and down-regulated genes in order to identify those which are expressed in only one species and, through background knowledge of the effects of the said gene, might suggest a mechanism of facilitated non-genotoxic carcinogenesis or protection. Of course, the situation is likely to be far more complex. Perhaps if there were one key gene protecting guinea pig from non-genotoxic effects and it was upregulated 50 times by PPs, the same gene might only be up-regulated five times in the rat. However, since both were noted to be upregulated, the importance of the gene may be overlooked. Just to complicate matters, a large change in expression does not necessarily mean a biologically important change. For example, what is the true relevance of gene Y which shows a 50-fold increase after a particular treatment, and gene Z which shows only a 5-fold increase? If one examines the literature one may find that historically, gene Y has often been shown to be up-regulated 40–60-fold by a number of unrelated stimuli—in light of this the 50-fold increas would appear less significant. However, the literature may show that gene Z has never be n recorded as having more than doubled in expression—which makes your 5-fold increase all the more exciting. Perhaps even more interesting is if that same 5-fold increase has only been seen in related neoplasms or following treatment with related chemicals.

Problems in using the differ ntial display approach

Differential display technology originally held promise of an easily obtainable 'fingerprint' of those genes which are up- or down-regulated in test animals/cells in a developmental process or following exposure to given stimuli. However, it has

become clear that the fingerprinting process, whilst still valid, is much too complex to be represented by a single technique profile. This is because all differential display techniques have common and/or unique technical problems which preclude the isolation and identification of all those genes which show changes in expression. Furthermore, there are important genetic changes related to disease development which differential expression analysis is simply not designed to address. An example of this is the presence of small deletions, insertions, or point mutations such as those seen in activated oncogenes, tumour suppressor genes and individual polymorphisms. Polymorphic variations, small though they usually are, are often regarded as being of paramount importance in explaining why some patients respond better than others to certain drug treatments (and, in logical extension, why some people are less affected by potentially dangerous xenobiotics/carcinogens than others). The identification of such point mutations and naturally occurring polymorphisms requires the subsequent application of sequencing, SSCP, DGGE or TGGE to the gene of interest. Furthermore, differential display is not designed to address issues such as alternatively spliced gene species or whether an increased abundance of mRNA is a result of increased transcription or increased mRNA stability.

Conclusions

Perhaps the main advantage of open system differential display techniques is that they are not limited by extant theories or researcher bias in revealing genes which are differentially expressed, since they are designed to amplify all genes which demonstrate altered expression. This means that they are useful for the isolation of previously unknown genes which may turn out be useful biomarkers of a particular state or condition. At least one open system (SAGE) is also quantitative, thus eliminating the need to return to the original mRNA and carry out Northern/PCR analysis to confirm the result. However, the rapid progress of genome mapping projects means that over the next 5–10 years or so, the balance of experimental use will switch from open to closed differential display systems, particularly DNA arrays. Arrays are easier and faster to prepare and use, provide quantitative data, are suitable for high throughput analysis and can be tailored to look at specific signalling pathways or families of genes. Identification of all the gene sequences in human and common laboratory animals combined with improved DNA array technology, means that it will soon no longer be necessary to try to isolate differentially expressed genes using the technically more demanding open system approach. Thus, their main advantage (that of identifying unknown genes) will be largely eradicated. It is likely, therefore, that their sphere of application will be reduced to analysis of the less common laboratory species, since it will be some time yet before the genomes of such animals as zebrafish, electric eels, gerbils, crayfish and squid, for example, will be sequenced.

Of course, in the end the question will always remain: What is the functional/biological significance of the identified, differentially expressed genes? One persistent problem is understanding whether differentially expressed genes are a cause or consequence of the altered state. Furthermore, many chemicals, such as non-genotoxic carcinogens, are also mitogens and so genes associated with replication will also be upregulated but may have little or nothing to do with the

carcinogenic effect. Whilst differential display technology cannot hope to answer these questions, it does provide a springboard from which identification, regulatory and functional studies can be launched. Understanding the molecular mechanism of cellular responses is almost impossible without knowing the regulation and function of those genes and their condition (e.g. mutated). In an abstract sense, differential display can be likened to a still photograph, showing details of a fixed moment in time. Consider the Historian who knows the outcome of a battle and the placement and condition of the troops before the battle commenced, but is asked to try and deduce how the battle progressed and why it ended as it did from a few still photographs—an impossible task. In order to understand the battle, the Historian must find out the capabilities and motivation of the soldiers and their commanding officers, what the orders were and whether they were obeyed. He must examine the terrain, the remains of the battle and consider the effects the prevailing weather conditions exerted. Likewise, if mechanistic answers are to be forthcoming, the scientist must use differential display in combination with other techniques, such as knockout technology, the analysis of cell signalling pathways, mutation analysis and time and dose response analyses. Although this review has emphasized the importance of differential gene profiling, it should not be considered in isolation and the full impact of this approach will be strengthened if used in combination with functional genomics and proteomics (2-dimensional protein gels from isoelectric focusing and subsequent SDS electrophoresis and virtual 2D-maps using capillary electrophoresis). Proteomics is attracting much recent attention as many of the changes resulting in differential gene expression do not involve changes in mRNA levels, as decribed extensively herein, but rather protein–protein, protein–DNA and protein phosphorylation events which would require functional genomics or proteomic technologies for investigation.

Despite the limitations of differential display technology, it is clear that many potential applications and benefits can be obtained from characterizing the genetic changes that occur in a cell during normal and disease development and in respons to chemical or biological insult. In light of functional data, such profiling will provide a 'fingerprint' of each stage of development or response, and in the long term should help in the elucidation of specific and sensitive biomarkers for different types of chemical/biological exposure and disease states. The potential medical and therapeutic benefits of understanding such molecular changes are almost immeasurable. Amongst other things, such fingerprints could indicate the family or even specific type of chemical an individual has been exposed to plus the length and/or acuteness of that exposure, thus indicating the most prudent treatment. They may also help uncover differences in histologically identical cancers, provide diagnostic tests for the earliest stages of neoplasia and, again, perhaps indicate the most efficacious treatment.

The Human Genome Project will be completed early in the next century and the DNA sequence of all the human genes will be known. The continuing development and evolution of differential gene expression technology will ensure that this knowledge contributes fully to the understanding of human disease processes.

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IN PERSPECTIVE
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Microarrays and Toxicology: The Advent of Toxicogenomics

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The availability of genome-scale DNA sequence information and reagents has radically altered life-science research. This revolution has led to the development of a new scientific subdiscipline derived from a combination of the fields of toxicology and genomics. This subdiscipline, termed toxicogenomics, is concerned with the identification of potential human and environmental toxicants, and their putative mechanisms of action, through the use of genomics resources. One such resource is DNA microarrays or "chips," which allow the monitoring of the expression levels of thousands of genes simultaneously. Here we propose a general method by which gene expression, as measured by cDNA microarrays, can be used as a highly sensitive and informative marker for toxicity. Our purpose is to acquaint the reader with the development and current state of microarray technology and to present our view of the usefulness of microarrays to the field of toxicology. *Mol. Carcinog. 24:153–159, 1999.*

Key words: toxicology; gene expression; animal bioassay

INTRODUCTION

Technological advancements combined with intensive DNA sequencing efforts have generated an enormous database of sequence information over the past decade. To date, more than 3 million sequences, totaling over 2.2 billion bases [1], are contained within the GenBank database, which includes the complete sequences of 19 different organisms [2]. The first complete sequence of a free-living organism, *Haemophilus influenzae*, was reported in 1995 [3] and was followed shortly thereafter by the first complete sequence of a eukaryote, *Saccharomyces cervisiae* [4]. The development of dramatically improved sequencing methodologies promises that complete elucidation of the *Homo sapiens* DNA sequence is not far behind [5].

To exploit more fully the wealth of new sequence information, it was necessary to develop novel methods for the high-throughput or parallel monitoring of gene expression. Established methods such as northern blotting, RNAse protection assays, S1 nuclease analysis, plaque hybridization, and slot blots do not provide sufficient throughput to effectively utilize the new genomics resources. Newer methods such as differential display [6], high-density filter hybridization [7,8], serial analysis of gene expression [9], and cDNA- and oligonucleotide-based microarray "chip" hybridization [10–12] are possible solutions to this bottleneck. It is our belief that the microarray approach, which allows the monitoring of expression levels of thousands of genes simultaneously, is a tool of unprecedented power for use in toxicology studies.

Almost without exception, gene expression is altered during toxicity, as either a direct or indirect result of toxicant exposure. The challenge facing toxicologists is to define, under a given set of experimental conditions, the characteristic and specific pattern of gene expression elicited by a given toxicant. Microarray technology offers an ideal platform for this type of analysis and could be the foundation for a fundamentally new approach to toxicology testing.

MICROARRAY DEVELOPMENT AND APPLICATIONS

cDNA Microarrays

In the past several years, numerous systems were developed for the construction of large-scale DNA arrays. All of these platforms are based on cDNAs or oligonucleotides immobilized to a solid support. In the cDNA approach, cDNA (or genomic) clones of interest are arrayed in a multi-well format and amplified by polymerase chain reaction. The products of this amplification, which are usually 500- to 2000-bp clones from the 3′ regions of the genes of interest, are then spotted onto solid support by using high-speed robotics. By using this method, microarrays of up to 10 000 clones can be generated by spotting onto a glass substrate

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Abbreviations: PAH, polycyclic aromatic hydrocarbon; NIEHS, National Institute of Environmental Health Sciences.

[13,14]. Sample detection for microarrays on glass involves the use of probes labeled with fluorescent or radioactive nucleotides.

Fluorescent cDNA probes are generated from control and test RNA samples in single-round reverse-transcription reactions in the presence of fluorescently tagged dUTP (e.g., Cy3-dUTP and Cy5-dUTP), which produces control and test products labeled with different fluors. The cDNAs generated from these two populations, collectively termed the "probe," are then mixed and hybridized to the array under a glass coverslip [10,11,15]. The fluorescent signal is detected by using a custom-designed scanning confocal microscope equipped with a motorized stage and lasers for fluor excitation [10,11,15]. The data are analyzed with custom digital image analysis software that determines for each DNA feature the ratio of fluor 1 to fluor 2, corrected for local background [16,17]. The strength of this approach lies in the ability to label RNAs from control and treated samples with different fluorescent nucleotides, allowing for the simultaneous hybridization and detection of both populations on one microarray. This method eliminates the need to control for hybridization between arrays. The research groups of Drs. Patrick Brown and Ron Davis at Stanford University spearheaded the effort to develop this approach, which has been successfully applied to studies of *Arabidopsis thaliana* RNA [10], yeast genomic DNA [15], tumorigenic versus non-tumorigenic human tumor cell lines [11], human T-cells [18], yeast RNA [19], and human inflammatory disease–related genes [20]. The most dramatic result of this effort was the first published account of gene expression of an entire genome, that of the yeast *Saccharomyces cervisiae* [21].

In an alternative approach, large numbers of cDNA clones can be spotted onto a membrane support, albeit at a lower density [7,22]. This method is useful for expression profiling and large-scale screening and mapping of genomic or cDNA clones [7,22–24]. In expression profiling on filter membranes, two different membranes are used simultaneously for control and test RNA hybridizations, or a single membrane is stripped and reprobed. The signal is detected by using radioactive nucleotides and visualized by phosphorimager analysis or autoradiography. Numerous companies now sell such cDNA membranes and software to analyze the image data [25–27].

Oligonucleotide Microarrays

Oligonucleotide microarrays are constructed either by spotting prefabricated oligos on a glass support [13] or by the more elegant method of direct in situ oligo synthesis on the glass surface by photolithography [28–30]. The strength of this approach lies in its ability to discriminate DNA molecules based on single base-pair difference. This allows the application of this method to the fields of medical diagnostics, pharmacogenetics, and sequencing by hybridization as well as gene-expression analysis.

Fabrication of oligonucleotide chips by photolithography is theoretically simple but technically complex [29,30]. The light from a high-intensity mercury lamp is directed through a photolithographic mask onto the silica surface, resulting in deprotection of the terminal nucleotides in the illuminated regions. The entire chip is then reacted with the desired free nucleotide, resulting in selected chain elongation. This process requires only 4n cycles (where n = oligonucleotide length in bases) to synthesize a vast number of unique oligos, the total number of which is limited only by the complexity of the photolithographic mask and the chip size [29,31,32].

Sample preparation involves the generation of double-stranded cDNA from cellular poly(A)+ RNA followed by antisense RNA synthesis in an in vitro transcription reaction with biotinylated or fluor-tagged nucleotides. The RNA probe is then fragmented to facilitate hybridization. If the indirect visualization method is used, the chips are incubated with fluor-linked streptavidin (e.g., phycoerythrin) after hybridization [12,33]. The signal is detected with a custom confocal scanner [34]. This method has been applied successfully to the mapping of genomic library clones [35], to de novo sequencing by hybridization [28,36], and to evolutionary sequence comparison of the *BRCA1* gene [37]. In addition, mutations in the cystic fibrosis [38] and BRCA1 [39] gene products and polymorphisms in the human immunodeficiency virus-1 clade B protease gene [40] have been detected by this method. Oligonucleotide chips are also useful for expression monitoring [33] as has been demonstrated by the simultaneous evaluation of gene-expression patterns in nearly all open reading frames of the yeast strain *S. cerevisiae* [12]. More recently, oligonucleotide chips have been used to help identify single nucleotide polymorphisms in the human [41] and yeast [42] genomes.

THE USE OF MICROARRAYS IN TOXICOLOGY

Screening for Mechanism of Action

The field of toxicology uses numerous in vivo model systems, including the rat, mouse, and rabbit, to assess potential toxicity and these bioassays are the mainstay of toxicology testing. However, in the past several decades, a plethora of in vitro techniques have been developed to measure toxicity, many of which measure toxicant-induced DNA damage. Examples of these assays include the Ames test, the Syrian hamster embryo cell transformation assay, micronucleus assays, measurements of sister chromatid exchange and unscheduled DNA synthesis, and many others. Fundamental to all of these methods is the fact that toxicity is often preceded by, and results in, alterations in gene expression. In many cases, these changes in gene expression are a

far more sensitive, characteristic, and measurable endpoint than the toxicity itself. We therefore propose that a method based on measurements of the genome-wide gene expression pattern of an organism after toxicant exposure is fundamentally informative and complements the established methods described above.

We are developing a method by which toxicants can be identified and their putative mechanisms of action determined by using toxicant-induced gene expression profiles. In this method, in one or more defined model systems, dose and time-course parameters are established for a series of toxicants within a given prototypic class (e.g., polycyclic aromatic hydrocarbons (PAHs)). Cells are then treated with these agents at a fixed toxicity level (as measured by cell survival), RNA is harvested, and toxicant-induced gene expression changes are assessed by hybridization to a cDNA microarray chip (Figure 1). We have developed a custom DNA chip, called ToxChip v1.0, specifically for this purpose and will discuss it in more detail below. The changes in gene expression induced by the test agents in the model systems are analyzed, and the common set of changes unique to that class of toxicants, termed a toxicant signature, is determined.

This signature is derived by ranking across all experiments the gene-expression data based on relative fold induction or suppression of genes in treated samples versus untreated controls and selecting the most consistently different signals across the sample set. A different signature may be established for each prototypic toxicant class. Once the signatures are determined, gene-expression profiles induced by unknown agents in these same model systems can then be compared with the established signatures. A match assigns a putative mechanism of action to the test compound. Figure 2 illustrates this signature method for different types of oxidant stressors, PAHs, and peroxisome proliferators. In this example, the unknown compound in question had a gene-expression profile similar to that of the oxidant stressors in the database. We anticipate that this general method will also reveal cross talk between different pathways induced by a single agent (e.g., reveal that a compound has both PAH-like and oxidant-like properties). In the future, it may be necessary to distinguish very subtle differences between compounds within a very large sample set (e.g., thousands of highly similar structural isomers in a combinatorial chemistry library or peptide library). To generate these highly refined signatures, standard statistical clustering techniques or principal-component analysis can be used.

For the studies outlined in Figure 2, we developed the custom cDNA microarray chip ToxChip v1.0.

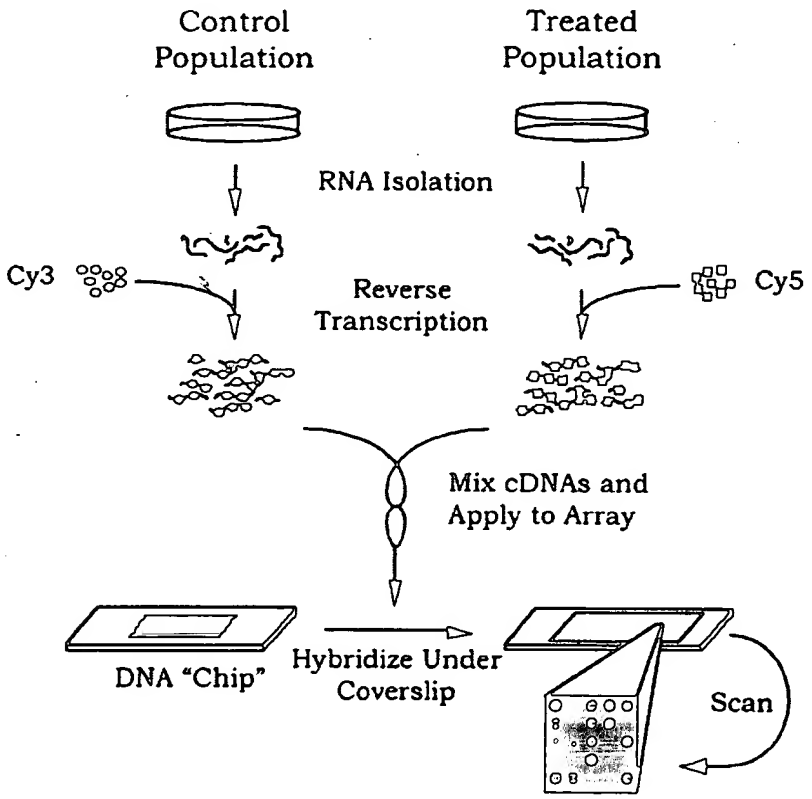

Figure 1. Simplified overview of the method for sample preparation and hybridization to cDNA microarrays. For illustrative purposes, samples derived from cell culture are depicted, although other sample types are amenable to this analysis.

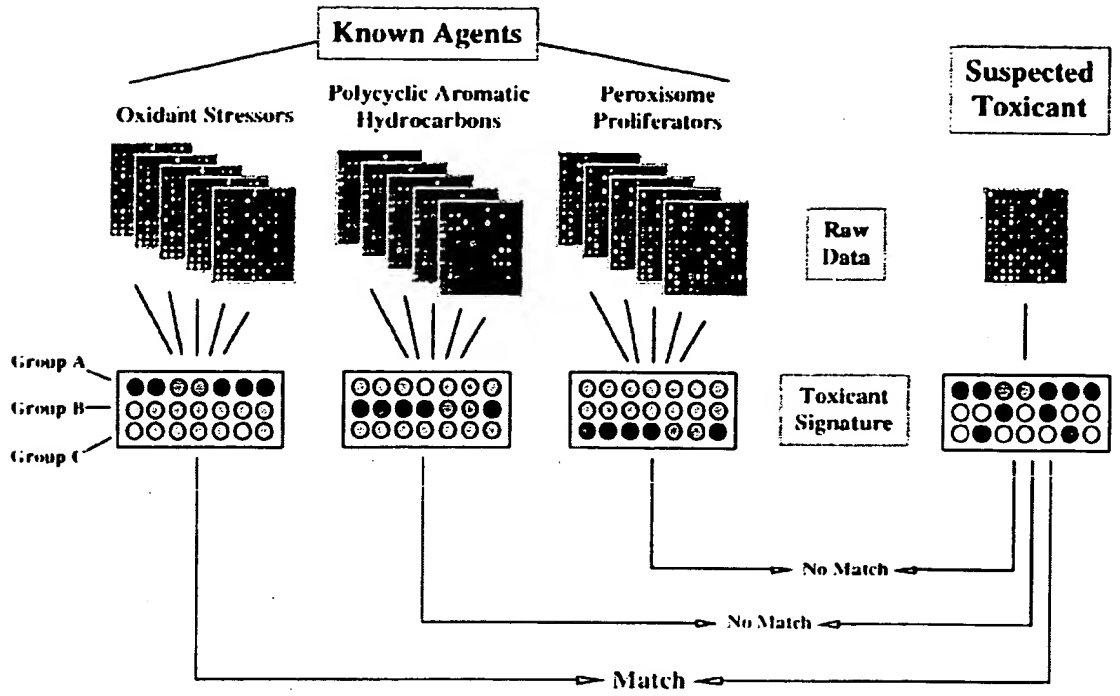

Figure 2. Schematic representation of the method for identification of a toxicant's mechanism of action. In this method, gene-expression data derived from exposure of model systems to known toxicants are analyzed, and a set of changes characteristic to that type of toxicant (termed the toxicant signature) is identified. As depicted, oxidant stressors produce consistent changes in group A genes (indicated by red and green circles), but not group B or C genes (indicated by gray circles). The set of gene-expression changes elicited by the suspected toxicant is then compared with these characteristic patterns, and a putative mechanism of action is assigned to the unknown agent.

The 2090 human genes that comprise this subarray were selected for their well-documented involvement in basic cellular processes as well as their responses to different types of toxic insult. Included on this list are DNA replication and repair genes, apoptosis genes, and genes responsive to PAHs and dioxin-like compounds, peroxisome proliferators, estrogenic compounds, and oxidant stress. Some of the other categories of genes include transcription factors, oncogenes, tumor suppressor genes, cyclins, kinases, phosphatases, cell adhesion and motility genes, and homeobox genes. Also included in this group are 84 housekeeping genes, whose hybridization intensity is averaged and used for signal normalization of the other genes on the chip. To date, very few toxicants have been shown to have appreciable effects on the expression of these housekeeping genes. However, this housekeeping list will be revised if new data warrant the addition or deletion of a particular gene. Table 1 contains a general description of some of the different classes of genes that comprise ToxChip v1.0.

When a toxicant signature is determined, the genes within this signature are flagged within the database. When uncharacterized toxicants are then screened, the data can be quickly reformatted so that blocks of genes representing the different signatures are displayed [11]. This facilitates rapid, visual interpretation of data. We are also developing ToxChip v2.0 and chips for other model systems, including rat, mouse, *Xenopus*, and yeast, for use in toxicology studies.

Animal Models in Toxicology Testing

The toxicology community relies heavily on the use of animals as model systems for toxicology testing. Unfortunately, these assays are inherently expensive, require large numbers of animals and take a long time to complete and analyze. Therefore, the National Institute of Environmental Health Sciences (NIEHS), the National Toxicology Program, and the toxicology community at large are committed to reducing the number of animals used, by developing more efficient and alternative testing methodologies. Although substantial progress has been made in the development of alternative methods, bioassays are still used for testing endpoints such as neurotoxicity, immunotoxicity, reproductive and developmental toxicology, and genetic toxicology. The rodent cancer bioassay is a particularly expensive and time-consuming assay, as it requires almost 4 yr, 1200 animals, and millions of dollars to execute and analyze [43]. In vitro experiments of the type outlined in Figure 2 might provide evidence that an unknown

Table 1. ToxChip v1.0: A Human cDNA Microarray Chip Designed to Detect Responses to Toxic Insult

Gene category	No. of genes on chip
Apoptosis	72
DNA replication and repair	99
Oxidative stress/redox homeostasis	90
Peroxisome proliferator responsive	22
Dioxin/PAH responsive	12
Estrogen responsive	63
Housekeeping	84
Oncogenes and tumor suppressor genes	76
Cell-cycle control	51
Transcription factors	131
Kinases	276
Phosphatases	88
Heat-shock proteins	23
Receptors	349
Cytochrome P450s	30

*This list is intended as a general guide. The gene categories are not unique, and some genes are listed in multiple categories.

agent is (or is not) responsible for eliciting a given biological response. This information would help to select a bioassay more specifically suited to the agent in question or perhaps suggest that a bioassay is not necessary, which would dramatically reduce cost, animal use, and time.

The addition of microarray techniques to standard bioassays may dramatically enhance the sensitivity and interpretability of the bioassay and possibly reduce its cost. Gene-expression signatures could be determined for various types of tissue-specific toxicants, and new compounds could be screened for these characteristic signatures, providing a rapid and sensitive in vivo test. Also, because gene expression is often exquisitely sensitive to low doses of a toxicant, the combination of gene-expression screening and the bioassay might allow the use of lower toxicant doses, which are more relevant to human exposure levels, and the use of fewer animals. In addition, gene-expression changes are normally measured in hours or days, not in the months to years required for tumor development. Furthermore, microarrays might be particularly useful for investigating the relationship between acute and chronic toxicity and identifying secondary effects of a given toxicant by studying the relationship between the duration of exposure to a toxicant and the gene-expression profile produced. Thus, a bioassay that incorporates gene-expression signatures with traditional endpoints might be substantially shorter, use more realistic dose regimens, and cost substantially less than the current assays do.

These considerations are also relevant for branches of toxicology not related to human health and not using rodents as model systems, such as aquatic toxicology and plant pathology. Bioassays based on the flathead minnow, *Daphnia*, and *Arabadopsis* could also be improved by the addition of microarray analysis. The combination of microarrays with traditional bioassays might also be useful for investigating some of the more intractable problems in toxicology research, such as the effects of complex mixtures and the difficulties in cross-species extrapolation.

Exposure Assessment, Environmental Monitoring, and Drug Safety

The currently used methods for assessment of exposure to chemical toxicants are based on measurement of tissue toxin levels or on surrogate markers of toxicity, termed biomarkers (e.g., peripheral blood levels of hepatic enzymes or DNA adducts). Because gene expression is a sensitive endpoint, gene expression as measured with microarray technology may be useful as a new biomarker to more precisely identify hazards and to assess exposure. Similarly, microarrays could be used in an environmental-monitoring capacity to measure the effect of potential contaminants on the gene-expression profiles of resident organisms. In an analogous fashion, microarrays could be used to measure gene-expression endpoints in subjects in clinical trials. The combination of these gene-expression data and more established toxic endpoints in these trials could be used to define highly precise surrogates of safety.

Gene-expression profiles in samples from exposed individuals could be compared to the profiles of the same individuals before exposure. From this information, the nature of the toxic exposure can be determined or a relative clinical safety factor estimated. In the future it may also be possible to estimate not only the nature but the dose of the toxicant for a given exposure, based on relative gene-expression levels. This general approach may be particularly appropriate for occupational-health applications, in which unexposed and exposed samples from the same individuals may be obtainable. For example, a pilot study of gene expression in peripheral-blood lymphocytes of Polish coke-oven workers exposed to PAHs (and many other compounds) is under consideration at the NIEHS. An important consideration for these types of studies is that gene expression can be affected by numerous factors, including diet, health, and personal habits. To reduce the effects of these confounding factors, it may be necessary to compare pools of control samples with pools of treated samples. In the future it may be possible to compare exposed sample sets to a national database of human-expression data, thus eliminating the need to provide an unexposed sample from the same individual. Efforts to develop such a national gene-expression database are currently under way [44,45]. However, this national database approach will require a better understanding of genome-wide gene expression across the highly diverse human population and of the effects of environmental factors on this expression.

Alleles, Oligo Arrays, and Toxicogenetics

Gene sequences vary between individuals, and this variability can be a causative factor in human diseases of environmental origin [46,47]. A new area of toxicology, termed toxicogenetics, was recently developed to study the relationship between genetic variability and toxicant susceptibility. This field is not the subject of this discussion, but it is worthwhile to note that the ability of oligonucleotide arrays to discriminate DNA molecules based on single base-pair differences makes these arrays uniquely useful for this type of analysis. Recent reports demonstrated the feasibility of this approach [41,42]. The NIEHS has initiated the Environmental Genome Project to identify common sequence polymorphisms in 200 genes thought to be involved in environmental diseases [48]. In a pilot study on the feasibility of this application to the Environmental Genome Project, oligonucleotide arrays will be used to resequence 20 candidate genes. This toxicogenetic approach promises to dramatically improve our understanding of interindividual variability in disease susceptibility.

FUTURE PRIORITIES

There are many issues that must be addressed before the full potential of microarrays in toxicology research can be realized. Among these are model system selection, dose selection, and the temporal nature of gene expression. In other words, in which species, at what dose, and at what time do we look for toxicant-induced gene expression? If human samples are analyzed, how variable is global gene expression between individuals, before and after toxicant exposure? What are the effects of age, diet, and other factors on this expression? Experience, in the form of large data sets of toxicant exposures, will answer these questions.

One of the most pressing issues for array scientists is the construction of a national public database (linked to the existing public databases) to serve as a repository for gene-expression data. This relational database must be made available for public use, and researchers must be encouraged to submit their expression data so that others may view and query the information. Researchers at the National Institutes of Health have made laudable progress in developing the first generation of such a database [44,45]. In addition, improved statistical methods for gene clustering and pattern recognition are needed to analyze the data in such a public database.

The proliferation of different platforms and methods for microarray hybridizations will improve sample handling and data collection and analysis and reduce costs. However, the variety of microarray methods available will create problems of data compatibility between platforms. In addition, the near-infinite variety of experimental conditions under which data will be collected by different laboratories will make large-scale data analysis extremely difficult. To help circumvent these future problems, a set of standards to be included on all platforms should be established. These standards would facilitate data entry into the national database and serve as reference points for cross-platform and inter-laboratory data analysis.

Many issues remain to be resolved, but it is clear that new molecular techniques such as microarray hybridization will have a dramatic impact on toxicology research. In the future, the information gathered from microarray-based hybridization experiments will form the basis for an improved method to assess the impact of chemicals on human and environmental health.

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Expression profiling in toxicology — potentials and limitations

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Abstract

Recent progress in genomics and proteomics technologies has created a unique opportunity to significantly impact the pharmaceutical drug development processes. The perception that cells and whole organisms express specific inducible responses to stimuli such as drug treatment implies that unique expression patterns, molecular fingerprints, indicative of a drug's efficacy and potential toxicity are accessible. The integration into state-of-the-art toxicology of assays allowing one to profile treatment-related changes in gene expression patterns promises new insights into mechanisms of drug action and toxicity. The benefits will be improved lead selection, and optimized monitoring of drug efficacy and safety in pre-clinical and clinical studies based on biologically relevant tissue and surrogate markers.

Keywords: Proteomics; Genomics; Toxicology

1. Introduction

The majority of drugs act by binding to protein targets, most to known proteins representing enzymes, receptors and channels, resulting in effects such as enzyme inhibition and impairment of signal transduction. The treatment-induced perturbations provoke feedback reactions aiming to compensate for the stimulus, which almost always are associated with signals to the nucleus, resulting in altered gene expression. Such gene expression regulations account for both the pharmacological action and the toxicity of a drug and can be visualized by either global mRNA or global protein expression profiling. Hence, for each individual drug, a characteristic gene regulation pattern, its molecular fingerprint, exists which bears valuable information on its mode of action and its mechanism of toxicity.

Gene expression is a multistep process that results in an active protein (Fig. 1). There exist numerous regulation systems that exert control at and after the transcription and the translation step. Genomics, by definition, encompasses the quantitative analysis of transcripts at the mRNA level, while the aim of proteomics is to quantify gene expression further down-stream, creating a snapshot of gene regulation closer to ultimate cell function control.

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2. Global mRNA profiling

Expression data at the mRNA level can be produced using a set of different technologies such as DNA microarrays, reverse transcript imaging, amplified fragment length polymorphism (AFLP), serial analysis of gene expression (SAGE) and others. Currently, DNA microarrays are very popular and promise a great potential. On a typical array, each gene of interest is represented either by a long DNA fragment (200–2400 bp) typically generated by polymerase chain reaction (PCR) and spotted on a suitable substrate using robotics (Schena et al., 1995; Shalon et al., 1996) or by several short oligonucleotides (20–30 bp) synthesized directly onto a solid support using photolabile nucleotide chemistry (Fodor et al., 1991; Chee et al., 1996). From control and treated tissues, total RNA or mRNA is isolated and reverse transcribed in the presence of radioactive or fluorescent labeled nucleotides, and the labeled probes are then hybridized to the arrays. The intensity of the array signal is measured for each gene transcript by either autoradiography or laser scanning confocal microscopy. The ratio between the signals of control and treated samples reflect the relative drug-induced change in transcript abundance.

3. Global protein profiling

Global quantitative expression analysis at the protein level is currently restricted to the use of two-dimensional gel electrophoresis. This technique combines separation of tissue proteins by isoelectric focusing in the first dimension and by sodium dodecyl sulfate slab gel electrophoresis-based molecular weight separation on the second, orthogonal dimension (Anderson et al., 1991). The product is a rectangular pattern of protein spots that are typically revealed by Coomassie Blue, silver or fluorescent staining (Fig. 2). Protein spots are identified by mass spectrometry following generation of peptide mass fingerprints (Mann et al., 1993) and sequence tags (Wilkins et al., 1996). Similar to the mRNA approach, the ratio between the optical density of spots from control and treated samples are compared to search for treatment-related changes.

4. Expression data analysis

Bioinformatics forms a key element required to organize, analyze and store expression data from either source, the mRNA or the protein level. The overall objective, once a mass of high-quality

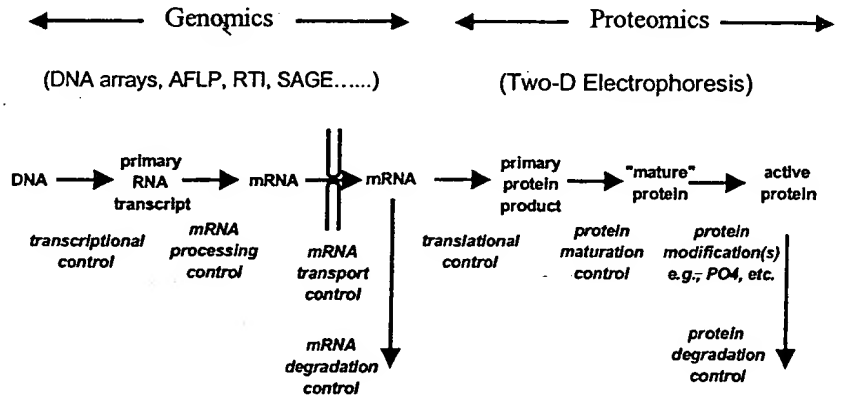

Fig. 1. Production of an active protein is a multistep process in which numerous regulation systems exert control at various stages of expression. Molecular fingerprints of drugs can be visualized through expression profiling at the mRNA level (genomics) using a variety of technologies and at the protein level (proteomics) using two-dimensional gel electrophoresis.

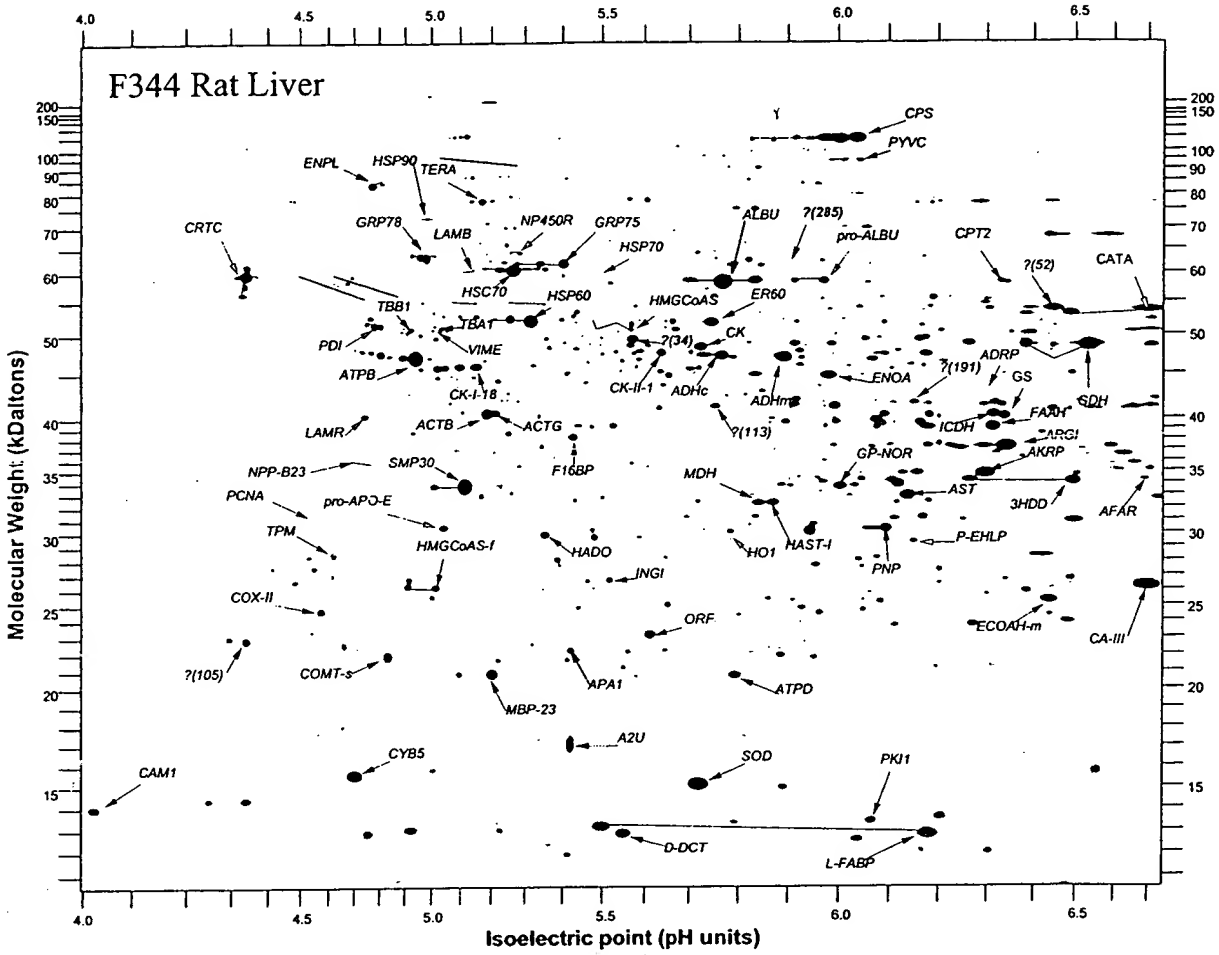

Fig. 2. Computerized representation of a Coomassie Blue stained two-dimensional gel electrophoresis pattern of Fischer F344 rat liver homogenate.

quantitative expression data has been collected, is to visualize complex patterns of gene expression changes, to detect pathways and sets of genes tightly correlated with treatment efficacy and toxicity, and to compare the effects of different sets of treatment (Anderson et al., 1996). As the drug effect database is growing, one may detect similarities and differences between the molecular fingerprints produced by various drugs, information that may be crucial to make a decision whether to refocus or extend the therapeutic spectrum of a drug candidate.

5. Comparison of global mRNA and protein expression profiling

There are several synergies and overlaps of data obtained by mRNA and protein expression analysis. Low abundant transcripts may not be easily quantified at the protein level using standard two-dimensional gel electrophoresis analysis and their detection may require prefractionation of samples. The expression of such genes may be preferably quantified at the mRNA level using techniques allowing PCR-mediated target amplifi-

cation. Tissue biopsy samples typically yield good quality of both mRNA and proteins; however, the quality of mRNA isolated from body fluids is often poor due to the faster degradation of mRNA when compared with proteins. RNA samples from body fluids such as serum or urine are often not very 'meaningful', and secreted proteins are likely more reliable surrogate markers for treatment efficacy and safety. Detection of post-translational modifications, events often related to function or nonfunction of a protein, is restricted to protein expression analysis and rarely can be predicted by mRNA profiling. Information on subcellular localization and translocation of proteins has to be acquired at the level of the protein in combination with sample prefractionation procedures. The growing evidence of a poor correlation between mRNA and protein abundance (Anderson and Seilhamer, 1997) further suggests that the two approaches, mRNA and protein profiling, are complementary and should be applied in parallel.

6. Expression profiling and drug development

Understanding the mechanisms of action and toxicity, and being able to monitor treatment efficacy and safety during trials is crucial for the successful development of a drug. Mechanistic insights are essential for the interpretation of drug effects and enhance the chances of recognizing potential species specificities contributing to an improved risk profile in humans (Richardson et al., 1993; Steiner et al., 1996b; Aicher et al., 1998). The value of expression profiling further increases when links between treatment-induced expression profiles and specific pharmacological and toxic endpoints are established (Anderson et al., 1991, 1995, 1996; Steiner et al. 1996a). Changes in gene expression are known to precede the manifestation of morphological alterations, giving expression profiling a great potential for early compound screening, enabling one to select drug candidates with wide therapeutic windows reflected by molecular fingerprints indicative of high pharmacological potency and low toxicity (Arce et al., 1998). In later phases of drug development, surrogate markers of treatment efficacy and toxicity can be applied to optimize the monitoring of pre-clinical and clinical studies (Doherty et al., 1998).

7. Perspectives

The basic methodology of safety evaluation has changed little during the past decades. Toxicity in laboratory animals has been evaluated primarily by using hematological, clinical chemistry and histological parameters as indicators of organ damage. The rapid progress in genomics and proteomics technologies creates a unique opportunity to dramatically improve the predictive power of safety assessment and to accelerate the drug development process. Application of gene and protein expression profiling promises to improve lead selection, resulting in the development of drug candidates with higher efficacy and lower toxicity. The identification of biologically relevant surrogate markers correlated with treatment efficacy and safety bears a great potential to optimize the monitoring of pre-clinical and clinical trails.

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Subject: RE: [Fwd: T xic logy Chip]
Date: Mon. 3 Jul 2000 08:09:45 -0400
From: "Afshari.Cynthia" <afshari@niehs.nih.gov>
To: "'Diana Hamlet-Cox'" <dianahc@incyte.com>

You can see the list of clones that we have on our 12K chip at
http://manuel.niehs.nih.gov/maps/guest/clonesrch.cfm
We selected a subset of genes (2000K) that we believed critical to tox response and basic cellular processes and added a set of clones and ESTs to this. We have included a set of control genes (80-) that were selected by the NHGRI because they did not change across a large set of array experiments. However, we have found that some of these genes change significantly after tox treatments and are in the process of looking at the variation of each of these 80- genes across our experiments.
Our chips are constantly changing and being updated and we hope that our data will lead us to what the toxchip should really be.
I hope this answers your question.
Cindy Afshari

> ----------
> *From: Diana Hamlet-Cox*
> *Sent: Monday, June 26, 2000 8:52 PM*
> *To: afshari@niehs.nih.gov*
> *Subject: [Fwd: Toxicology Chip]*
>
> *Dear Dr. Afshari,*
>
> *Since I have not yet had a response from Bill Grigg, perhaps he was not the right person to contact.*
>
> *Can you help me in this matter? I don't need to know the sequences, necessarily, but I would like very much to know what types of sequences are being used, e.g., GPCRs (more specific?), ion channels, etc.*
>
> *Diana Hamlet-Cox*
>
> *-------- Original Message --------*
> *Subject: Toxicology Chip*
> *Date: Mon, 19 Jun 2000 18:31:48 -0700*
> *From: Diana Hamlet-Cox <dianahc@incyte.com>*
> *Organization: Incyte Pharmaceuticals*
> *To: grigg@niehs.nih.gov*
>
> *Dear Colleague:*
>
> *I am doing literature research on the use of expressed genes as pharmacotoxicology markers, and found the Press Release dated February 29, 2000 regarding the work of the NIEHS in this area. I would like to know if there is a resource I can access (or you could provide?) that would give me a list of the 12,000 genes that are on your Human ToxChip Microarray. In particular, I am interested in the criteria used to select sequences for the ToxChip, including any control sequences included in the microarray.*
>
> *Thank you for your assistance in this request.*
>
> *Diana Hamlet-Cox, Ph.D.*
> *Incyte Genomics, Inc.*
>
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